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THE MEDIAN EYE OF LIMULUS: AN ULTRAVIOLET RECEPTOR

By George Wald and James M. Krainin*

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Communicated October 14, 1963

Of the major groups of animals that have well-formed lateral eyes, capable of resolving images—the arthropods, cephalopod molluscs, and vertebrates—only the cephalopods have failed to develop also median eyes. In many arthropods these take the form of paired median ocelli, each with a single lens that casts an image upon a more or less well-organized retina. In vertebrates the parietal eye ("pineal eye") is such a structure.

The functions of all these organs are fairly obscure. The retina is usually too small and coarse-grained and the lens too primitive to make them seem well-adapted for image vision. Though the exposure of median eyes to light evokes electrical activity,[1, 3, 4] it has proved difficult to demonstrate associated behavioral responses. It has been suggested for this reason that median eyes may stimulate hormone production or secretion in the central nervous system, and that in insects they may modulate the level of brain activity.

In the horseshoe crab, *Limulus*, a pair of ocelli lies at the base of the median frontal spine. In young animals such as were used in the present experiments, with carapaces 7–9 cm wide, the ocelli are about 0.5 mm in diameter; even in animals 40 cm wide they are only 1–2 mm in diameter, no bigger than a pinhead.

Figure 1 shows the cross section of a young median ocellus of *Limulus*, according to Demoll.[2] Over the ocellus, the cuticle forms a cornea which is fused with an almost spherical lens. The shape of the latter suggests that it may pinch off later as a separate structure. An area of epidermis is modified behind the lens to form the vitreous. The retina contains receptor cells (retinulae) resembling those of the compound eye, similarly gathered together in groups of up to 6–8, as in ommatidia. Also as in the compound eye, the retinulae can sometimes be seen to bear along one border a rhabdomere, a solid organelle that probably contains the visual pigment. A young ocellus may contain 50–80 retinulae,[3] each ending in an optic nerve fiber. The optic nerves from both ocelli fuse together and pass as a single nerve around one side of the digestive tract to the brain.[4]

Demoll also described a network of epidermal cells that form inner and outer limiting membranes bordering both surfaces of the retina, with many cross-bridges that interpenetrate the clumps of retinulae, carving the retina into numerous segments. The general arrangement of these cells resembles that of the

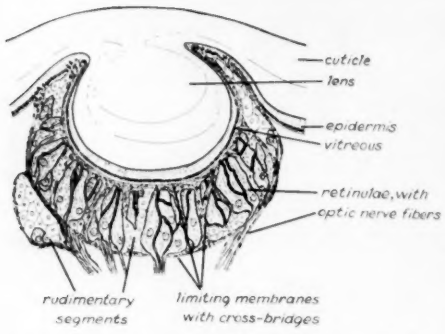

FIG. 1.—Cross section of a median ocellus of *Limulus*, according to Demoll.[2] An almost spherical lens is fused with the cuticle. The epidermis differentiates in the eye region to form the vitreous, some cells of which contain a few pigment granules. The epidermis also gives rise to a network of cells which compose the outer and inner limiting membranes, enclosing the retina, with many connecting strands that interpenetrate the visual cells. The latter, the retinulae, are gathered together in clumps resembling ommatidia of the compound eye. Each retinula ends in an optic nerve fiber. Blocks of relatively undifferentiated tissue, as in the compound eye, form so-called rudimentary segments. Such a rudimentary segment as shown at the lower left, which is wholly detached, Demoll thought capable of migration along the optic nerve. It seems to contain a functional retinula and optic fiber(s), and such units, embedded in the optic nerve, may account for its observed sensitivity to light.[4]

Müller cells of the vertebrate retina.

Portions of the retina are composed of spongy, relatively undifferentiated tissue—also as in the compound eye—forming so-called rudimentary segments. Such segments may contain what appear to be functional retinulae and optic fibers. One such segment (Fig. 1, left) appears wholly detached from the remainder of the retina. Demoll believed such units capable of migration down (why not also up?) the optic nerve, and such units, embedded in the optic nerve, probably account for Waterman's curious observation that it is sensitive to light.[4]

Wulff and Pandazi[3] recorded electroretinograms (ERG's) from retinas isolated from the median eyes of *Limulus*, and found them to vary with the intensity and duration of light much as does the ERG of the compound eye. Later, Waterman[4] recorded action potentials ("spikes") from small bundles of optic nerve fibers. On exposing the eye to light there is a short latent period, then a burst of impulses, beginning at a high frequency and falling to a lower frequency maintained as long as the light is on.

In the present experiments we have measured the spectral sensitivities of the median and lateral eyes of young *Limulus*. The sensitivity of the median eyes is concentrated in the near ultraviolet, with a high peak at about 360 mμ. There is also a secondary maximum at about 530–535 mμ, close to the sensitivity maximum of the compound eye, but in the ocellus less than 1 per cent as high as the ultraviolet maximum. The responses associated with these two maxima in the ocellus differ in character and vary independently of each other, so that they must involve different visual pigments, and probably also different receptors and central connections.

Apparatus and Procedures.—The light of a 100-watt zirconium arc lamp was projected with quartz lenses through a grating monochromator (Bausch and Lomb; 250-mm focal length, grating 1200 lines/mm, linear dispersion 3.3 mμ/mm). With 1.5-mm slits this instrument transmitted a wave band 5 mμ wide throughout the spectrum. A photographic shutter at the exit slit regulated the exposure, and a quartz lens projected the image of the exit slit on a pair of circular neutral wedges, rotating in opposite directions so as to compensate each other. One wedge was of quartz, the other of optical glass, and both were made by evaporating a metal film of graded density on these supporting materials (Eastman Kodak Co.). The wedges together yielded a range of densities of a little more than 5. The light transmitted by them was focused by another quartz lens on the eye.

The energy distribution of this optical system was calibrated with a Moll thermopile (Kipp and Zonen) of 80 elements, generating a mean emf of 0.083 μvolts/μwatt/sq cm. The voltage was measured with a Keithley 150A electronic microvolt-ammeter, reading down to 0.1 μvolt. The wedge was calibrated with a Welch Densichron in a series of overlapping ranges, at each 20 mμ from 320–680 mμ. Fortunately its attenuation relative to the open position was about the same at all wavelengths, so that one calibration curve sufficed. The calibration was finally rendered in terms of relative numbers of quanta per flash reaching the eye.

Electroretinograms were measured with a Tektronix *502* dual-beam oscilloscope and a Grass *P5* AC preamplifier with a half-amplitude frequency of 0.1 cps, run from a Grass regulated power supply. Electroretinograms were photographed with a Polaroid camera mounted on the oscilloscope (Dumont). For long exposures of the eye to light, a Grass *P6* DC preamplifier was used.

Young *Limulus* 7–9 cm wide were held by pins stuck through the borders of the shell on a platform of paraffin wax. A wad of absorbent cotton soaked in sea water, packed under them, kept them moist throughout the experiment. The surfaces of the lateral eyes and ocelli were gently scraped with a razor blade to make better contact with the active electrode, and a small hole made in the shell with a needle and stuffed with cotton provided the contact for the indifferent electrode. The electrodes were cotton wicks, held in glass tubes pulled out at one end into capillary tips and filled with sea water. Contact was through nonpolarizable silver—silver chloride electrodes. The animals were not harmed by the experiment, and some were used again and again during several weeks. All measurements of spectral sensitivity involved determining the relative numbers of photons per flash at various wavelengths needed to evoke a peak amplitude in the ERG of 70 μvolts.

Measurements.—Figure 2 shows the spectral sensitivities of both the median and lateral eyes of dark-adapted animals. The solid circles show measurements on both organs in a single animal; the open circles are from two other animals. The spectral sensitivity is the reciprocal of the relative number of quanta at each wavelength needed to evoke a peak amplitude of 70 μvolts in the ERG, and here is plotted logarithmically. For clearer comparison, the scale of log sensitivities is lowered one unit in the data from the compound eye; their maximal sensitivity is in fact 0.85 log unit (about seven times) *higher* than that of the ocelli.

The spectral sensitivity of the lateral eyes had been measured earlier.[5] It is maximal at about 525 mμ, and appears to be based upon a single visual pigment with λ_{max} about 520 mμ.[6]

The spectral sensitivity of the ocelli is concentrated in a narrow band in the near ultraviolet, maximal at about 360 mμ. They have also a secondary peak

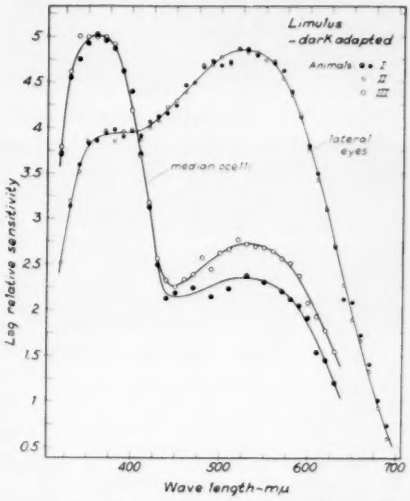

FIG. 2.—Comparison of the spectral sensitivities of median ocelli and lateral compound eyes in young dark-adapted *Limulus*. Solid circles show measurements involving both organs in one animal; open circles show similar data from two other animals. The sensitivity, here plotted logarithmically, is the reciprocal of the number of photons at each wavelength needed to excite a constant peak amplitude in the ERG (70 μvolts). Exposures, 45 msec. The scale of log sensitivity has been lowered one unit in the data for the compound eyes; their maximal sensitivity is about 0.85 log unit (about 7 times) higher than that of the ocelli. The lateral eyes are maximally sensitive at about 525 mμ; the ocelli at about 360 mμ, with a small secondary peak at about 530–535 mμ. The difference in relative heights of the peaks at 360 and 530 mμ in the ocelli from two animals is one indication that two different visual pigments, and perhaps two different types of receptors, are involved.

of sensitivity at 530–535 mμ, close therefore to the main peak in the compound eyes. This sensitivity band not only lies at a slightly longer wavelength than that of the lateral eyes, but is distinctly broader, mainly because its long wavelength limb descends less rapidly. There is therefore no present reason to conclude that both these sensitivity bands go with the same visual pigment, though possibly this is so, and differences in the transmission of light by ocular structures, or some other physical factors, cause the observed differences.

In the two ocelli that yielded the data in Figure 2, the secondary peak is 2.25 and 2.65 log units lower than—0.22–0.55 per cent as high as—the ultraviolet peak. In an arithmetic plot of these data it would scarcely show at all.

The difference in relative heights of the 530- and 360-mμ peaks in the ocelli of two animals shows that these peaks vary independently of each other. They probably therefore involve two different visual pigments, and perhaps two different receptor types. We shall have more to say of this below.

In the same vein the presence of a sharp shoulder in the spectral sensitivity curve for the lateral eyes near 360 mμ might be taken to indicate the presence of some 360-mμ pigment in this organ also. This is indeed possible, yet this shoulder is more likely to be caused principally by a rapid decline of transmission by the ocular structures—cornea, lens, and rhabdome—at lower wavelengths.

Figure 3 shows the results of an experiment intended to examine further the apparent independence of the peaks at 360 and 530 mμ. The spectral sensitivity of a single median ocellus, having been measured in the dark-adapted condition, was remeasured with the ocellus continuously adapted to near ultraviolet light. This adaptation depressed the sensitivity at 360 mμ 1.75 log units (56 times), while depressing the sensitivity at 530 mμ only 0.13 log unit (1.35 times). The mechanisms that go with these two sensitivity peaks therefore adapt independently of each other, a further indication that they must involve different visual pigments, and perhaps different receptors.

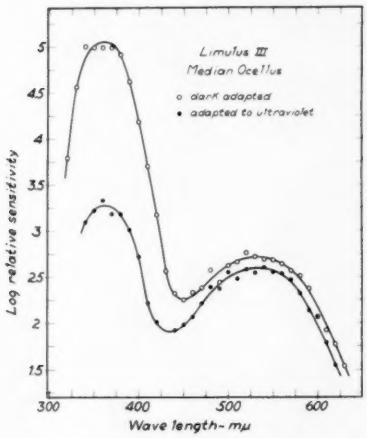

FIG. 3.—Spectral sensitivity of the median ocellus in a single animal while dark-adapted (open circles), and while continuously exposed to near ultraviolet light (solid circles; Corning filter 5860: 330–385 mμ, with peak transmission at 365 mμ). Adaptation to this ultraviolet light depresses the sensitivity at 360 mμ 1.75 log units (56 times), while depressing it at 530 mμ only 0.13 log unit (1.35 times). Clearly these sensitivity peaks adapt independently of each other, an indication that they involve different visual pigments and perhaps different receptors.

Another step in the same argument is shown in Figure 4. Here electroretinograms are recorded at 550 and 370 mμ from a single ocellus, a few minutes apart and under circumstances altogether the same except that the intensities of the stimulus were adjusted at the two wavelengths so as to yield equal amplitudes of ERG. Such comparisons were made a number of times, always with the result shown. At 370 mμ the ERG is a simple, relatively symmetrical monophasic wave, with a latency of about 60 msec, and reaching peak ampli-

tude about 115 msec after the onset of the stimulus. At 550 mμ the ERG is highly asymmetrical, the peak is reached sooner (in about 85 msec), and the response declines over a broad hump.

These differences in the wave form of the ERG in the visible and ultraviolet suggest strongly that two different receptors, probably with different central connections, are involved. It might be possible to achieve such different response patterns from a single receptor cell, but intrinsically this is much less likely.

In Figure 5 electroretinograms are compared from a median ocellus and a compound eye of the same animal. DC amplification permitted the measurement of the response to a relatively long stimulus—0.8 sec—without distortion. The ERG's from both organs are very similar except for one feature. The ERG from the lateral eye declines from its peak amplitude to a low steady-state level, maintained as long as the light is on, dropping back to the base-line 40–80 msec after the light goes off. The lateral eye exhibits this behavior at all levels of response (cf. ref. 7).

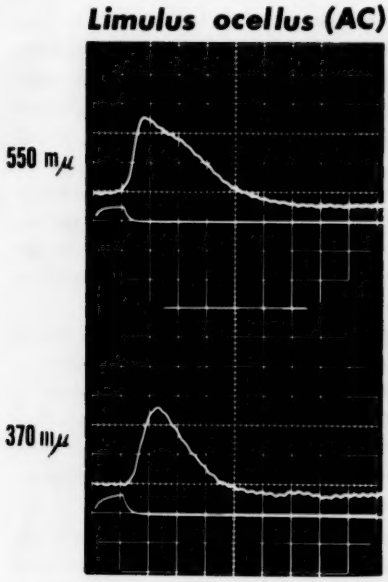

FIG. 4.—Electroretinograms from a median ocellus at 550 and 370 mμ. The heights of the ERG's have been matched to facilitate comparison. The differences of wave form shown are typical. Coupled with the existence of independent sensitivity peaks at these wavelengths, they suggest that different receptor units are involved. AC amplication; sensitivity 140 μvolts/division; sweep speed 50 msec/div.; half-time of exposure, recorded below each record, 45 msec.

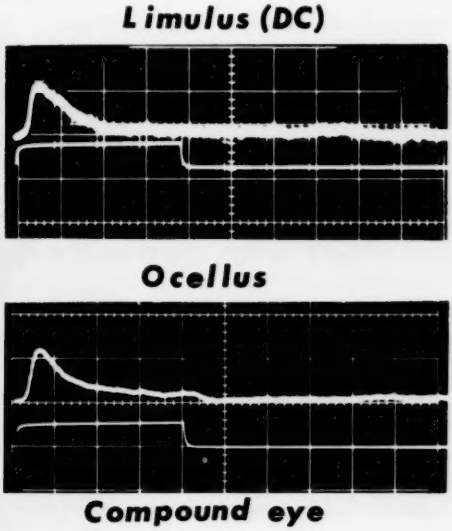

FIG. 5.—Electroretinograms from a median ocellus and a lateral compound eye of the same animal. Responses have been matched in height to facilitate comparison. They are similar but for one feature: the response of the compound eye falls from its peak to a lower value, maintained as long as the stimulus continues, then after a short added interval falling back to the base-line; whereas the response of the ocellus falls back to the base-line while the light is still on, and no further change is recorded when the light goes off. DC amplification; exposures about 0.8 sec (recorded below each ERG); stimulus 550 mμ; sweep speed 0.2 sec/division; sensitivity: top, 80 μvolts/div.; bottom, 200 μvolts/div.

In the median ocellus, however, the response falls from its peak amplitude back

to the base-line even while the stimulus continues. On turning off the light there is no further change. We have observed this type of behavior in all our trials.

It should not be concluded that because the ERG becomes inappreciable in continuous light the organ ceases altogether to respond. Waterman's records of action potentials in optic nerve fibers from the ocellus[4] show that a low level of firing continues as long as the light is on. Nevertheless, the failure of the ERG to maintain a measurable level of response in continued illumination or to signal the turning off of the light suggests that the ocellus is more concerned with signaling sudden increases than decreases in illumination.

Discussion.—The remarkable feature of the ocellar response is its concentration of sensitivity in the ultraviolet. Goldsmith and Ruck[8] have measured the spectral sensitivities of dorsal ocelli in cockroaches and worker honeybees. In the roach the spectral sensitivity is concentrated in the visible region, with a peak at about 500 mμ. A low level of sensitivity extends throughout the ultraviolet to about 300 mμ, but with no additional peak and no indication that a second type of receptor is involved. In the bee the median ocellus exhibits two peaks of sensitivity roughly comparable in height, at 490 and 335–340 mμ. As in the *Limulus* ocellus, these probably involve two types of receptor, as evidenced by differences in the wave form of the ERG in the two spectral regions, and differences in the relative sensitivities at 335 and 490 mμ in different individuals.

Yanase and Fujimoto[9] have recently reported the extraction of a visual pigment from the medial ocelli of bees, the difference spectrum of which has λ_{max} 450 mμ. This λ_{max} does not coincide with either sensitivity peak found in bee ocelli by Goldsmith and Ruck, and the status of this pigment is questionable on other grounds: the surprisingly large amount of pigment extracted from relatively little tissue, and the fact that it requires nearly an hour in bright light to bleach. Possibly this is a screening rather than a visual pigment.

It has long been known that the compound eyes of a number of insects are highly sensitive to ultraviolet light. Walther and Dodt[10] have reported a dominant peak at 341 mμ in the fly *Calliphora*, accompanied by nearly as high peaks at 500 and 630 mμ. In the cockroach *Periplaneta* they found a main peak at 500–510 mμ and a secondary peak at 340–370 mμ, which adapted independently of each other. In worker bees Goldsmith[11] found a dominant peak of sensitivity at 535 mμ, accompanied by a minor band at 345 mμ, apparently associated with different receptor units.

Recently the spectral sensitivities of single visual cells have been measured with microelectrodes in the compound eyes of the fly *Calliphora*[12] and the drone bee.[13] In *Calliphora* three types of receptors were found, each with a sensitivity maximum in the visible region (524, 490, 468 mμ) and an additional maximum in the ultraviolet at 345 mμ. (It should be noted that these maxima do not agree well, particularly in the visible, with those reported by Walther and Dodt.[10]) In the drone bee two receptor types were found, peaking in sensitivity at about 447 and 340 mμ. The latter apparently corresponds with the ultraviolet receptor found by Goldsmith in the worker bee.

The *Limulus* ocellus is an organ designed primarily for the reception of ultraviolet light, its sensitivity in the visible seeming negligible by comparison. It may be argued that this is a laboratory view, going with artificial stimulation of the eye

in air. *Limulus* is a bottom animal, found often in fairly turbid waters. The ultraviolet component of sunlight might be so feeble, and its attenuation by water so great, as to more than make up for the large disparity in sensitivity of the ocellus in the ultraviolet and visible.

Apart from turbidity of the water, however, about which little can be said, this is apparently not the case. The energy of sunlight reaching the earth is about $^1/_4$ as intense at 360 mμ as at 530 mμ, and even at a depth of 20 m of clear water this fraction has fallen only to about $^1/_6$.[14] The two animals measured in Figure 2 are about 180–450 times as sensitive at 360 as at 530 mμ. In daylight at the surface, therefore, 360 mμ should be 40–110 times as effective as 530 mμ, and under 20 m of water 30–70 times as effective as 530 mμ in stimulating the ocelli of these animals. The ocellus seems therefore to be primarily an ultraviolet receptor in natural environments. On the other hand, its relatively insensitive receptor units for the visible spectrum may serve quite different and important functions.

What those functions may be is still wholly problematical. Baylor and Smith[15] have made the significant observation that in the fresh-water cladoceran *Daphnia magna* the behavior patterns associated with vertical migration are governed by the ocelli, and exhibit a special sensitivity to violet and ultraviolet light. There is now good reason to look for specific responses in *Limulus* that depend upon the stimulation of the ocelli by ultraviolet light, and perhaps—since they apparently involve other receptor units—still other responses mediated by the ocelli in the visible spectrum.

* This investigation was supported in part with funds from the National Science Foundation and the Office of Naval Research. J. M. K. held a Summer Fellowship (1963) from the Harvard Medical School.

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*HISTONES AND DIFFERENTIATION**

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Communicated by John A. Moore, October 22, 1963

In 1950, Stedman and Stedman[1] suggested that histones may function in the nucleus as gene suppressors. The recent work of Huang and Bonner[2] on pea seedlings supports the idea that histone suppresses gene activity, since DNA that is free of histone synthesizes far more messenger RNA than does DNA complexed with histone. Similarly, Allfrey *et al.*[3, 4] found that in calf thymus nuclei removal of histone appears to activate previously "repressed" DNA primers.

In 1953, Alfert and Geschwind[5] demonstrated that alkaline fast green could be used as a specific stain for nucleohistone. Their cytochemical studies, and those of Bloch and Godman,[6] showed that the distribution of DNA and histone is the same in each nucleus and that the synthesis of one parallels that of the other. In embryos of the mouse, however, Alfert[7] found no fast green staining in interphase cleavage nuclei and suggests that this lack of stainability is due to masking of the basic groups of the histones by other substances, since the mitotic chromosomes are stained. Histone staining appeared in the blastula nuclei.

In early snail embryos, Bloch and Hew[8] found that both nuclei and mitotic chromosomes failed to stain with alkaline fast green. Positive chromatin staining appeared at gastrulation. They described these results as a change in nuclear histones from "cleavage histones" to "adult histones." Bloch[9] suggests a connection between changes in nuclear histones and nuclear function. If histones control the expression of the DNA with which they are associated, such changes would be expected in early embryos. Bloch[9] states that there is no histone staining during interphase, but mitotic chromosomes stain, in the early embryos of the frog, *Rana pipiens*. In a study of normal *R. pipiens* embryos and hybrids between *R. pipiens* ♀ × *R. sylvatica* ♂, which cease development as early gastrulae, Horn[10] found that the nuclei did not stain with alkaline fast green until the onset of gastrulation.

If the appearance of histone staining in nuclei at gastrulation is correlated with the onset of differentiation, embryos which fail to differentiate should not show positive alkaline fast green staining of their nuclei. Androgenetic hybrids between *R. pipiens* ♀ × *R. sylvatica* ♂ form normal blastulae and exhibit no visible evidence of differentiation, although they survive for some time.[11, 12] Their cells are incapable of differentiation, even when transplanted to normal embryos.[13] A study has been made of these embryos and their controls. It was found that alkaline fast green staining of nuclear histones occurred not only in all early gastrulae, but also in the arrested blastulae. Moreover, some nuclei of all late blastulae stained lightly, and even occasional nuclei of early blastulae stain. Mitotic chromosomes are stained in all embryos, including early blastulae—the earliest embryos studied.

Material and Methods.—General: Eggs from *Rana pipiens* were fertilized with sperm of *pipiens* males or *sylvatica* males. Porter's method[14] was used to remove the maternal chromosomes from some of the eggs. Three experiments were done. In the first, embryos were fixed when the controls *pip pip* were in stages[16] 7+, 9, 10, 12, 13–14, 16–18, 19–20, and 21. The (*pip*) *pip* androgenetic embryos exhibited the usual haploid syndrome. The (*pip*) *syl* androgenetic hybrid embryos became arrested blastulae.[12] Many of the *pip syl* diploid hybrids formed tiny, abnormal embryos;[15]

the rest were arrested as gastrulae. In the second experiment, embryos were fixed when the controls were in stages 9, 12, 13–14, and 19. Development of the four classes of embryos was the same as in the first experiment except that all of the *pip syl* diploid hybrids were arrested as gastrulae. In the third experiment, only *pip pip* early blastulae (stage 7) were used. No differences were found among the embryos fixed at the same stage, so all have been combined in Table 1.

TABLE 1

NUMBER OF EMBRYOS STUDIED AFTER ALKALINE FAST GREEN STAINING

	Stage of Control Embryos							
	Early blastulae stage 7–7+	Late blastulae stage 9	Early gastrulae stage 10	Late gastrulae stage 12	Early neurulae stage 13–14	Stage 16–18	Stage 19–20	Stage 21
pip pip	8	6	2	4	4			
(*pip*) *pip*	4	6	2	4	4			
pip syl	4	6	2	4	4	8	6	2
(*pip*) *syl*	4	6	2	4	4	6	6	

Fixation: Embryos were fixed in 4% formaldehyde (10% neutral formalin) in McIlvain's buffer at pH 7.0 as recommended[9] but for $1^1/_4$ hr only since longer fixation hardens yolk and makes sectioning difficult. (Penetration of the fixative is good, since the eggs are 1.8 mm in diameter and their jelly membranes were removed.) They were dehydrated for 10 min in each of the following: 30, 50, 70, 85, and 95% alcohol; for 1 hr in absolute alcohol; stored in methyl benzoate-parlodion[17] for 20–26 days; cleared in benzene and imbedded. (Methyl benzoate-parlodion improves the sectioning of yolky material.) In addition small pieces of adult *pipiens* liver were fixed and dehydrated in the same way and stored 16 days in methyl benzoate-parlodion before imbedding.

Sectioning and mounting: In order to obtain whole nuclei, the embryos were sectioned between 5–14 μ, depending upon their stage of development and whether they were diploid or haploid. Since nuclei are larger in early embryos than in late, and diploid nuclei are larger than haploid, younger embryos and diploid embryos were sectioned at greater thicknesses than older embryos and haploid embryos. Sectioning just slightly greater than the size of the nuclei is desirable, since the pigment granules accumulate about the nuclei in blastulae and early gastrulae and obscure them in thick sections. Liver sections were cut at 10 μ and routinely mounted on all slides with the sections of the embryos as controls for staining. Since subsequent bleaching and hot TCA extraction often removed sections, the slides were coated with Haupt's adhesive instead of albumen, and the sections spread on 2% formaldehyde and dried for at least 24 hr in a 40°C oven saturated with the fumes from an open container of 40% formaldehyde.

Staining procedures: 1. *Alfert and Geschwind's method*[5] for alkaline fast green staining was precisely followed. The sections were extracted with 5% TCA at 90°C for 15 min and, after washing out the TCA, stained for 30 min with a 0.1% aqueous solution of the acid dye Fast Green FCF (Allied Chemical, National Aniline Division, 93% dye content) adjusted to pH 8.0–8.1. All embryos in Table 1 were stained and studied.

That staining of the nuclei with alkaline fast green occurred only after removal of nucleic acids by TCA was tested as follows: additional sections from 42 of the 112 embryos studied were not hydrolyzed with TCA but otherwise processed as usual and were found to be completely unstained by fast green. (These embryos ranged from control stages 9–21.) Bleached sections from 27 embryos (control stages 9–21) not hydrolyzed with TCA were completely unstained in 15 cases and in 12 had a faint green tinge in the large yolk platelets. This color was generally visible only at low magnifications. Parenchyma cells of liver sections similarly treated were always colorless.

2. Additional sections of all four kinds of embryos fixed when the controls were early blastulae, late blastulae, and early neurulae (Table 1) were stained according to the *picric acid–bromphenol blue procedure* of Bloch and Hew[18] which, they say, results in specific staining of nuclear basic proteins. The sections were hydrolyzed in a saturated aqueous solution of picric acid at 60–61°C for 15–18 hr, rinsed in water, stained for 1 hr in 0.1% bromphenol blue at pH 2.3, rinsed in slightly alkaline 95% ethanol, and differentiated in 95% ethanol for $21^1/_4$–23 hr.

Unlike fast green, which only stained material after TCA extraction, some bromphenol blue staining occurred without prior picric acid hydrolysis. In all embryos, most nuclei were colorless but a few were faintly blue; the smaller yolk platelets were light blue and the larger medium blue. In the liver, nuclei were colorless or blue to purple, with colorless heterochromatin, the cytoplasm

light to moderately blue. These results indicate that protein not bound to DNA may also be stained with bromphenol blue.

After picric acid hydrolysis, bromphenol blue staining of nuclei was also variable. Since yolk platelet staining was so intense in 10–14 μ sections that nuclei were difficult to distinguish, the embryos sectioned at 5–10 μ were studied. In early blastulae some nuclei were homogeneous, blue or richly purple; others were pale to colorless. Prophases were pale, with almost colorless chromosomal threads; metaphase chromosomes were light purple in a light blue spindle, and anaphase chromosomes very pale blue. All stained more intensely in late blastulae. All yolk platelets were purple, so the purple nuclei were difficult to distinguish in blastulae and more difficult in neurulae and arrested embryos, where they were smaller. In the liver cells whole nuclei were deep purple and homogeneous, lighter purple with darker heterochromatin, or blue with purple heterochromatin. The cytoplasm was blue to purple and varied from light to dark.

Since some nuclei of early blastulae are intensely stained but the chromosomes lightly stained, bromphenol blue is probably staining protein not associated with chromosomes, as well as nucleohistone. Moreover, since spindle areas in early blastulae and the liver cytoplasm are blue, bromphenol blue does not appear to be a specific stain for nucleohistone in the present material.

In Horn's study[10] no nuclear staining of pre-gastrulae with bromphenol blue was found, although faintly stained chromosomes were present. Possibly a difference in the thickness of the sections studied may be responsible for the discrepancy of our observations.

To block lysine staining and reveal arginine-rich protein,[8] the *pip pip* sections only were acetylated, after picric acid hydrolysis, for 1 hr at 60°C in acetic anhydride containing about 1% glacial acetic acid and then stained with bromphenol blue. Nuclei were not stained; yolk platelets were colorless or light to medium blue. Thus, in blastulae and neurulae, nuclear staining with bromphenol blue is probably not due to arginine-rich histone, such as protamine, but may be due to lysine-rich protein, some of which is associated with the chromosomes. Yolk platelet staining is probably due to protein rich in lysine and arginine.[10, 29]

Acetylation of liver sections resulted in colorless nuclei, or pale blue nuclei with darker heterochromatin, and colorless cytoplasm. Thus, bromphenol blue staining of nucleoplasm and heterochromatin may be due to lysine- and arginine-rich protein, and of cytoplasm to lysine-rich protein.

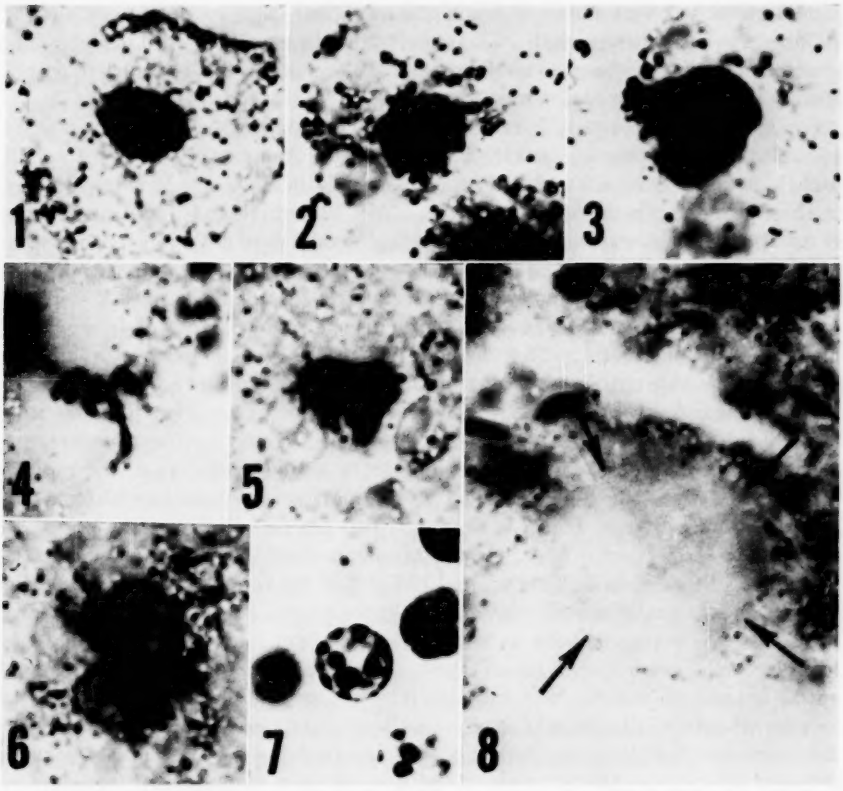
3. To test if fast green staining at other pH values is similar to that of bromphenol blue, unbleached sections of (*pip*) *pip* at stages 7, 9, 10, and 12, and of liver were processed according to the Alfert-Geschwind method but were stained with fast green at pH 8.6, 7.3, 6.05, and 4.6. Unbleached sections of *pip pip* at stages 7, 9, and 13, and of liver were stained at pH 2.3.

At pH 8.6 and 7.3, staining is similar to that at the usual pH of 8.0–8.1. At pH 6.05, embryonic nuclei and yolk platelets were colorless to pale green without prior TCA extraction, and after extraction the nuclei were stained so much like the medium green yolk platelets that, except at stage 12, they were indistinguishable. At pH 4.6 without prior TCA extraction, the embryonic nuclei and yolk platelets were pale to medium green; after extraction the nuclei were indistinguishable among the dark green yolk platelets. At both pH 6.05 and 4.6, liver nuclei were colorless and the cytoplasm colorless to pale green without prior TCA extraction. After extraction the nuclei were dark green, with darker nucleoli and heterochromatin, and the cytoplasm medium green. These results are somewhat similar to those after picric acid–bromphenol blue.

At pH 2.3, blastulae nuclei were a homogeneous dark green; stage 13 nuclei were blue-green, with visible chromatin, whether previously extracted with TCA or not. Yolk platelets were medium green without prior TCA extraction, and darker green after extraction. Without TCA extraction, liver nuclei were dark green with light heterochromatin; after TCA extraction the heterochromatin was also dark green. With or without TCA extraction the liver cytoplasm was spruce green. Hence there is a large amount of nonbasic protein in nuclei and yolk platelets of blastulae and gastrulae, and in the nuclei and cytoplasm of liver cells. Moreover, the homogeneous staining of blastulae nuclei suggests they have a larger amount of nonbasic protein than the smaller, stage 13 nuclei and liver nuclei.

Bleaching: Before either the alkaline fast green or bromphenol blue procedure, additional sections were treated with hydrogen peroxide; this bleaches the pigment granules from a dark brown to a golden yellow and makes it possible to see the nuclei within these lighter aggregates of granules. The sections were rehydrated to 70% ethanol and bleached in 10% H_2O_2 in 63% ethanol for $16^1/_2$–20 hr. So far as could be seen, bleaching did not affect the subsequent staining.

Results.—Alkaline fast green staining of nuclei and chromosomes: As was expected from Horn's study,[10] the nuclei in all early gastrulae were pale green to a clear, but not intense, green (Fig. 1). But surprisingly, the arrested (*pip*) *syl* blastulae fixed at the same time also had green nuclei (Fig. 2). In all these embryos the mitotic chromosomes were green. The nuclei were green (Fig. 3) in all embryos fixed at later stages (Table 1). At stage 12 the larger nuclei of the vegetal hemisphere



FIGS. 1–8.—All the photomicrographs are of alkaline fast green stained material taken through a red filter and magnified ×2000. (1) Nucleus of (*pip*) *pip* early gastrula; bleached section. (2) Nucleus of (*pip*) *syl* arrested blastula; unbleached section. (3) Nucleus of *pip pip* early neurula; unbleached section. (4) Metaphase chromosomes of *pip pip* early blastula; bleached section. (5) Anaphase chromosomes of *pip pip* late blastula; bleached section. (6) Nucleus of *pip pip* late blastula; bleached section. (7) Nucleus of *pip pip* adult liver; bleached section. (8) Nucleus of *pip pip* early blastula; bleached section; arrows indicate boundary of nucleus.

were lighter in color than the smaller nuclei of the animal hemisphere. When controls were stage 18, some of the nuclei of the arrested *pip syl* and (*pip*) *syl* were vesiculated and light green or condensed and fragmented into dark green droplets. More nuclei were involved in these changes as the embryos became older and moribund.

In all early and late blastulae mitotic chromosomes were stained (Figs. 4 and 5).

In all late blastulae some nuclei stained pale green (Fig. 6). When *pip pip* and *pip syl* were sectioned at 12 μ and (*pip*) *pip* at 10 μ and not bleached, interphase nuclei with their diffuse chromatin appeared chartreuse, due to the surrounding pigment granules, whereas the more condensed chromatin of prophases was green. In some of the (*pip*) *pip* the pigment granules so obscured the nuclei that any color in them could not be determined with surety in unbleached sections, but were ascertained to be chartreuse when bleached. In bleached 6-μ sections of (*pip*) *pip* and bleached 8-μ sections of *pip pip*, *pip syl*, and (*pip*) *syl*, fast green nuclear staining was easily determined. Therefore, failure to see any stained nuclei in late blastulae is due to the numerous pigment granules surrounding the nuclei and obscuring them in thicker sections.

Some nuclei of some early blastulae were stained faintly green. In two *pip pip* sectioned at 14 μ, some nuclei were faintly green or chartreuse (Fig. 8). In eight early blastulae sectioned at 12 μ, an occasional, faintly stained nucleus was seen in one *pip pip*, one *pip syl*, and one (*pip*) *syl*. In 10 sectioned at 6–10 μ, no staining of interphase nuclei was discernible. Although nuclei were not stained, some were opaque and discernible, and nuclear areas were recognizable as large, clear spaces surrounded by pigment granules. Yet in all early blastulae the mitotic chromosomes were green (Fig. 4), and even in the embryos sectioned at 6 μ, early prophases had pale green chromatin. Thus, in early blastulae, histone staining of interphase nuclei is discernible only in embryos which are cut at greater thicknesses.

When one compares the sizes of nuclei from early embryos (Figs. 6 and 8) with those of later embryos (Figs. 1–3) and of liver (Fig. 7), it is obvious that there is a marked decrease in size during development. Actual diameters of the *pip pip* nuclei photographed (except a different ectodermal nucleus of the late blastula was measured, since that of Fig. 6 is asymmetrical) are as follows: early blastula—15.5 μ, late blastula—8.5 μ, early neurula—7.8 μ, liver—5.9 μ. Their respective volumes are 1930 μ^3, 316 μ^3, 248 μ^3, and 108 μ^3. If the volume of the liver nucleus is taken as 1, the ratio of their respective volumes is 18:3:2.3:1. Thus, if the early blastula nucleus were to stain as intensely as the liver nucleus, it would have to have 18 $\times$ the amount of histone present. If the same amount of histone is present as in the liver nucleus (and this amount[5, 9, 19] in dividing nuclei is expected to be approximately 1–2$\times$ that of the liver nucleus), fast green staining will approach the limit of visibility.[20] As the volumes of the nuclei of the developing embryos decrease in size, the histone present will become more concentrated, hence more intensely stained with fast green and increasingly visible.

Data[21] on nuclear volumes of these embryos show a wide range of values at each developmental stage and a marked decrease in volume from early blastula to mid-gastrula stages. Subtelny and Bradt[22] found the volumes of 75 endodermal late blastula nuclei of *R. pipiens* varied from 697 to 2185 μ^3. When such nuclei are transplanted into an uncleaved egg, their volumes may increase up to 3315 μ^3. Dasgupta[23] found the diameter of the ♂ pronucleus to be 15 μ and that of the ♀ pronucleus to be 15–18 μ, a volume range of 1765–3060 μ^3. Although no volume measurements of fused pronuclei or of nuclei of cleavage stages of *pipiens* have been published, from the preceding data it can be deduced that the nuclei of embryos younger than early blastulae will be larger and would not be expected to stain visibly with fast green. It is now obvious why nuclei of early blastulae are visibly

stained only in thicker sections. These nuclei are very large and are cut in thinner sections. Since fast green is barely visible in a whole nucleus, the shorter pathway of a cut nucleus would show no visible color.[20]

As noted earlier, in those embryos where interphase nuclei did not appear to stain with fast green, mitotic stages from very early prophases to late telophases were stained. Thus, it seems that as the histone is no longer diffused throughout the nucleus but concentrated into the chromosomes, it becomes visible. It was also observed that the chromosomes stain with increasing intensity from early blastula to later stages, and this appears to be associated with a shortening of the chromosomes as development proceeds. The chromosomes of early blastulae appear to be longer than those of later stages. Di Berardino[24] has shown that the chromosomes of early gastrulae are from 1.3–1.9× as long as those from the tail tips of young larvae. Thus, chromosomes of later stages should appear more intensely stained, since the histone is more concentrated in the shorter chromosomes. Interphase nuclei of these same early blastulae stain with the Feulgen reaction for DNA light pink, but the chromosomes are brilliant red. Thus, staining of either the DNA or the histone of nuclei and chromosomes gives a similar picture.

Alkaline fast green staining of yolk platelets: The yolk platelets at the blastula stage are distributed in a gradient of increasing size, such that small yolk platelets are found in the animal hemisphere and large yolk platelets are in the majority in the vegetal hemisphere, although small yolk platelets are also present. As noted earlier, without TCA extraction, yolk platelets do not stain with alkaline fast green. After TCA extraction, fast green stains the large yolk platelets of the vegetal hemisphere green, and the small yolk platelets of the animal hemisphere light green to colorless. The intensity of the color varies with the size of the yolk platelet and the thickness of the section; in 6-μ sections even large yolk platelets are light green. Electron microscopic studies[25–28] have shown that yolk platelets possess a hexagonally shaped main body, a superficial layer, and an outer membrane. With the light microscope these components are clearly visible in some large yolk platelets. The main body stains with fast green (sometimes appearing lighter in the center); the superficial layer is usually colorless but may be faintly green; and the outer membrane appears as a dark line.

Horn[10] found variation in the intensity of staining of individual yolk platelets in early cleavage stages and little or no staining in the rapidly differentiating cells of post-gastrula embryos. Amino acid analyses of yolk protein showed it to be rich in lysine and arginine. He characterized yolk protein as extranuclear histone and suggested its transfer from the yolk platelets to the nuclei might be responsible for the altered staining pattern of pre- and post-gastrula cells. But the altered staining pattern of nuclei is explicable on physical grounds, as has been demonstrated in the present study.

Staining of the large yolk platelets of the arrested *pip syl* gastrulae and (*pip*) *syl* blastulae does not seem to change until they are moribund, when it is less intense. In *pip pip* and (*pip*) *pip* neurulae (stage 13–14) the yolk platelets in the epidermis are colorless or almost colorless, in the neutral plate and mesoderm colorless or faintly green or green, and green in the endoderm. As in blastulae, more intense staining seemed directly correlated with greater size of the yolk platelets and since small yolk platelets are present in ectodermal cells, these differentiating cells have

the palest yolk platelets. Thus, a causal relationship between differentiation and loss of yolk platelet staining could not be determined in these embryos.

Apparently in yolk platelets, as in nuclei, TCA hydrolysis makes available the amino groups of the basic amino acids for staining with fast green. Horn[10] suggests that the protein of the yolk platelets may be associated with RNA, which would be extracted by TCA, thus freeing the protein for staining.

RNA is extracted from sections hydrolyzed in 1 *N* HCl at 60°C for a short period of time, but DNA is not removed. To test whether yolk platelet staining resulted from the removal of RNA only, sections of *pip pip* embryos at stages 7, 9, 10, and 12 were hydrolyzed for 2, 5, 8, or 12 min, and of (*pip*) *pip* embryos of the same stages for 2 or 5 min in HCl and then stained with alkaline fast green. Only the smaller yolk platelets were lightly stained after 2 or 5 min hydrolysis, with no increase in intensity with longer hydrolysis. Thus, the intense staining of the yolk platelets after TCA extraction seems not to be due to removal of RNA, though possibly the light staining of the small yolk platelets is. Recently, however, Wallace[29] has concluded that *pipiens* yolk platelets most likely contain no RNA *in situ*.

The cytoplasmic DNA of *pipiens* embryos may well be associated with the yolk platelets.[29–32] If so, the protein of the yolk platelets may be bound to this DNA, and fast green staining of the yolk platelets could occur in a manner similar to that of the nuclei.

It is also possible that the phosphoprotein and lipoprotein of the yolk platelet[33] are associated neither with nuclei acid nor any other component. However, since yolk platelets have a crystalline pattern, the amino groups of the basic amino acids may be linked to other groups (such as the phosphate of phosphoserine) and hot TCA may break this linkage and free the basic amino acids for fast green staining. Moreover, since the yolk protein is highly concentrated, the yolk platelet may stain with fast green due to the presence of large numbers of amino groups and not because it has a particularly basic protein.

Discussion.—Nucleohistone has been demonstrated in nuclei of mouse blastulae[7] and of Drosophila[35] at the 10th cleavage mitosis. Not only is it present in nuclei of early *pipiens* embryos but it is probably actively synthesized, since in late blastulae there is a marked incorporation of H^3-arginine and H^3-histidine into the nuclei within $2^1/_2$ hr after injection of either basic amino acid into the blastocoel (B. C. Moore, unpublished data).

If the histone associated with DNA is an inhibitor of gene action, it should be present in early embryonic nuclei, which are genetically particularly inactive. At least in numerous crosses in Amphibia[36] it has been repeatedly demonstrated that diploid and androgenetic hybrids develop normally to gastrulation, indicating that the genes brought into the egg by the sperm have little effect until that time. Moreover, changes in developmental rate of hybrids do not occur until shortly after gastrulation,[37] and paternal antigens appear about the same time.[38] The ratio of histone to DNA may be greater in metabolically inactive nuclei than in more active nuclei.[39, 40]

One might suggest the following hypothesis for gene action and the role of histone in early development. Possibly before gastrulation the structural genes are, for the most part, "turned off,"[41] that is, they are closely associated with histone and are prevented from synthesizing messenger RNA. After gastrulation more and

more structural genes are "turned on," that is, their association with histone changes and they are able to synthesize messenger RNA.

Summary.—Histone is present in the nuclei and chromosomes of frog embryos not only after gastrulation but also at earlier stages. Nucleohistone is present in embryos which never differentiate but in which cell division continues and nuclei become smaller. In the larger nuclei of early embryos there may be no staining with alkaline fast green. This is thought to be a dilution effect. The picric acid–bromphenol blue procedure for nuclear basic proteins was also used. Staining of the yolk platelets is described and the role of histone in early development discussed.

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*REPRESSED AND ACTIVE CHROMATIN ISOLATED FROM INTERPHASE LYMPHOCYTES**

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These experiments involved the development of a procedure for isolating repressed and active chromatin from interphase calf thymus lymphocytes, and for studying the metabolism and morphology of such interphase chromosomal segments.

Up to 80 per cent of the deoxyribonucleic acid (DNA) of interphase thymus lymphocytes is inactive in messenger ribonucleic acid (RNA) synthesis,[1] and is apparently repressed,[2] correlating well with the differentiated state of these cells.[3] It has long been suggested that inactive chromosomal segments could be recognized cytologically during interphase and early prophase by their dense-staining characteristics, implying a condensed state (see ref. 4 for general review). Such condensed inactive chromosomal segments have been termed heterochromatin,[5–7] in contrast to segments in a more active, extended, diffusely staining state, termed euchromatin.

The heterochromatic sex-chromatin body of Barr[8] in female mammalian cells is composed of a segment of one X chromosome,[9–11] and the genes carried on this heterochromatic segment have been shown to be unexpressed in the phenotype of the cell.[12–14] The DNA of this heterochromatic X chromosome replicates later than that of other chromosomal segments,[15–17] and it has been shown that heterochromatic segments of interphase autosomes are much less active in RNA synthesis[6] and in DNA synthesis[18] than are euchromatic segments.

Much of the DNA of interphase thymus lymphocytes is visible cytologically as condensed, Feulgen-positive masses,[19, 20] resembling the heterochromatin of the inactive Barr sex-chromatin body. In this study it is shown that these condensed chromosomal segments can be isolated in mass from lymphocytes, that up to 80 per cent of the DNA of interphase lymphocytes is contained in these masses, and that the DNA, RNA, and protein metabolism of these condensed heterochromatin masses is decreased compared to that of the more-extended euchromatin fibrils.

Materials and Methods.—Previous isolations of chromatin: Chromatin represents interphase chromosomal segments and, as such, contains DNA, histones, nonhistone proteins, RNA, lipids, and perhaps polysaccharides.[21] The native association of such components is disrupted by marked departures of the isolation medium from isotonicity (salt concentration above 0.6 *M* or below 0.01 *M*) or from neutrality (above pH 8.0 or below pH 6.0),[22] imposing constraints upon the isolation procedure.[23] Previous methods of isolation of chromatin have utilized blending,[24, 25] grinding with sand[26] or with glass beads,[27] or ultrasonication[28, 29] of isolated nuclei that have been washed free of cytoplasm. In the method reported here, nuclear membranes, nuclear ribosomes, and soluble nuclear proteins were also removed before the isolation of chromatin fractions, and gelation was prevented throughout.

Isolation of interphase nuclei: A slight modification of the methods previously described[20] was used. Calf thymus lobes were brought to the laboratory in an ice-cold solution of 0.25 *M* sucrose within 1 hr of slaughtering. All subsequent operations other than incubation were performed at 2°C. The thymus of a single animal was selected, dissected free of adherent fat, connective tissue, and blood, and 100 gm of wet-weight tissue was finely minced. The mince was suspended in 1000 ml of 0.25 *M* sucrose–0.0033 *M* $CaCl_2$, and was homogenized at half speed in a Waring blendor 3 min. After filtering through cheesecloth, the homogenate was blended twice more for 3 min, with filtrations through flannelette, and was then centrifuged at 600 *g* for 7 min. The resultant nuclear pellet was washed once with 1000 ml of 0.25 *M* sucrose–0.0033 *M* $CaCl_2$, and was then suspended in 100 ml of an incubation medium of composition: 0.1875 *M* sucrose, 0.02 *M* glucose, 0.025 *M* Tris HCl, 0.0128 *M* NaCl, 0.0033 *M* $CaCl_2$, pH 7.1.

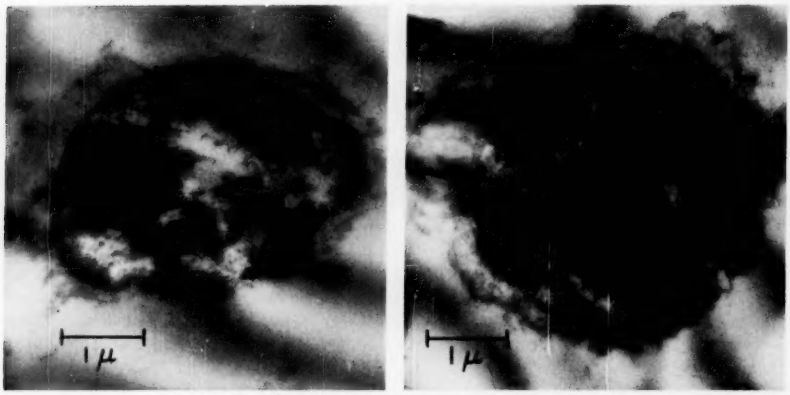
Incubation of nuclei: C^{14}-labeled precursors, such as thymidine-2-C^{14}, uridine-2-C^{14}, orotic acid-6-C^{14}, alanine-1-C^{14}, and lysine-1-C^{14} were added to the incubation suspensions, as shown in the legends to the tables, and were incubated at 37°C for 30 min in air with agitation. The incubation suspension was then added to 900 ml of cold incubation medium, and centrifuged at 600 *g* for 7 min.

Isolation of subnuclear fractions: The supernatant incubation medium contained cytoplasmic fragments and nuclear membranes,[31] which were removed in this and a second washing in 1000 ml of incubation medium. The washed nuclear pellet was extracted three times with 1000-ml volumes of 0.01 *M* Tris HCl–0.0033 *M* $CaCl_2$, pH 7.1, removing nuclear ribosomes and neutral proteins.[31] The easily decantable slurry following the third Tris extraction was removed, and the residual extracted nuclei were resuspended in 1000 ml of cation-free 0.25 *M* sucrose, allowed to stand for 10 min to permit swelling of the nuclei, and were then sonicated for 5 sec at 20,000 cycles per second and 7 amp on a Branson S-75 sonifier. Such brief sonication is much less than the 7–20 min employed in previous isolations of chromatin.[28, 29] Previous studies of the effect of sonication on isolated DNA showed that 5 sec exposure had minimal effects on the physical[32] and biological[33] properties of protein-free DNA, and that the native association of histone with DNA, as in chromatin, further stabilizes DNA against physical change.[32]

The suspension was sonicated in aliquots of 60 ml at a time, and the resulting sonicate was centrifuged at 100 *g* for 5 min, with the supernate being filtered through four layers of flannelette to remove any aggregated material. This filtrate was centrifuged at 1000 *g* for 10 min, and the pellet collected as condensed (hetero-)chromatin masses. The supernate was then centrifuged at 3000 *g* for 30 min, and the pellet collected as the intermediate fraction. The supernate obtained was then centrifuged at 78,000 *g* for 60 min, and this pellet collected as extended (eu-)chromatin fibrils. The resultant clear supernate was then made 10 mM in calcium chloride and centrifuged at 1000 *g* for 10 min. This pellet was collected as particles in 10 mM calcium. The measurement of protein, DNA, RNA, and radioactivity was as previously described.[31]

Electron microscopy: Electron micrographs of the whole tissue mince before the addition of solutions, and of the various centrifuge pellets, were obtained after fixation of the tissues for 15 min in 1% OsO_4,[20] dehydration in alcohol, embedding in Epon,[34] sectioning at 800-A thickness on a Cambridge-Huxley microtome, and staining for 15 min in 1% uranyl acetate.[20] Such staining is needed to reveal fully the condensed (hetero-)chromatin masses as seen by phase contrast or ultraviolet microscopy.[35, 36] A Phillips model EM-100 A electron microscope was used, yielding microscopic magnifications to ×20,000. Subsequent magnifications were obtained photographically.

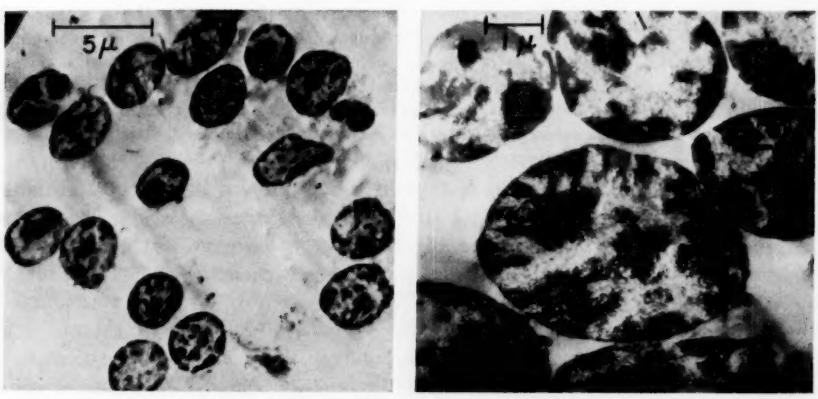
Results.—Electron micrographs of thymus tissue sampled before the addition of any preparative solutions (Figs. 1 and 2) reveal large masses of condensed (hetero-)chromatin arrayed peripherally toward the margins of the lymphocyte nucleus, with broad-based attachments to the nuclear membrane. These masses are seen at higher magnifications to be composed of a dense reticulum of 100-A fibrils, and are similar to those reported previously in thymus lymphocytes,[3, 19, 20] nucleated erythrocytes,[37] blood lymphocytes,[38] pancreas,[20, 39] liver,[40] von Kupfer cells and granulocytes,[41] lymphosarcoma cells,[36] Ehrlich ascites cells,[42] and as karyosomes[43] or chromocenters[5] in other well-differentiated cells. Fine strands of extended 50-A (eu-)chromatin fibrils are seen coursing between the



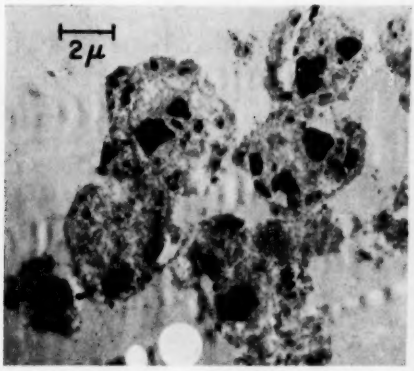
FIGS. 1 and 2.—Interphase calf thymus lymphocytes, before addition of preparative solutions. Masses of condensed (hetero-)chromatin contrast with fibrils of more extended (eu-)chromatin within the nucleus.

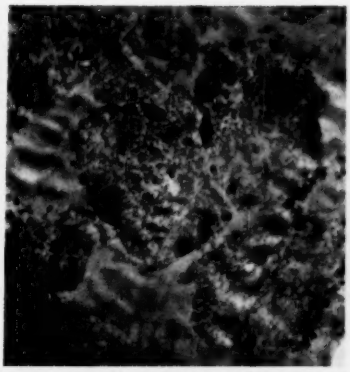
large condensed masses, and an occasional light-staining 0.5-μ nucleolus is seen, usually at the border of a condensed chromatin mass.[20]

Electron micrographs of isolated nuclei following extraction of nuclear ribosomes and neutral proteins (Figs. 3 and 4) reveal these same features, but when the extracted nuclei are resuspended in cation-free isotonic sucrose (Figs. 5 and 6), the nuclei swell to twice their normal size, the extended chromatin fibrils are spread, and these are seen to course from the periphery of the condensed chromatin masses.[44, 45] Following brief sonication and isolation of the condensed (hetero-) chromatin masses (Figs. 7 and 8), their basic structure and dimensions remain unaltered, with some fragmented euchromatin fibrils still attached. Isolated euchromatin fibrils (Fig. 9) are seen to be free of condensed chromatin masses. The isolated particles in 10 mM calcium (Fig. 10) appear as aggregates of particles somewhat resembling those of isolated nucleoli[29] or their fragments. The intermediate

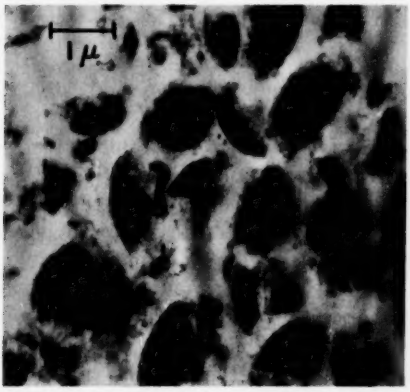


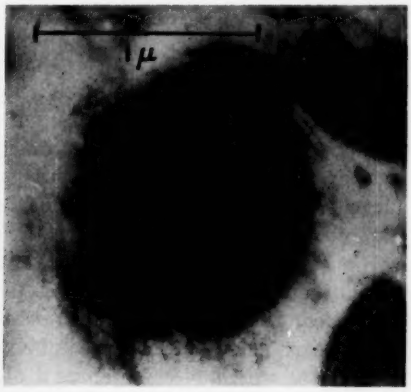
FIGS. 3 and 4.—Isolated lymphocyte nuclei after extraction of nuclear ribosomes. Masses of condensed (hetero-)chromatin, fibrils of more extended (eu-)chromatin, and an occasional light-staining nucleolus.





FIGS. 5 and 6.—Swollen nuclei in cation-free isotonic sucrose. Extended (eu-)chromatin fibrils are seen attached to the condensed (hetero-)chromatin masses, with fibril dispersion distally.





FIGS. 7 and 8.—Isolated condensed (hetero-)chromatin masses. A small number of euchromatic fragments remain attached to the masses.

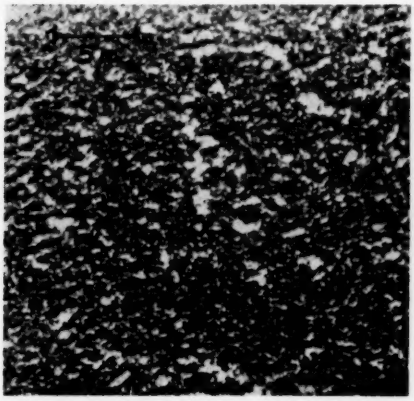


FIG. 9.—Isolated extended (eu-)chromatin fibrils. No condensed (hetero-)chromatin masses are found.

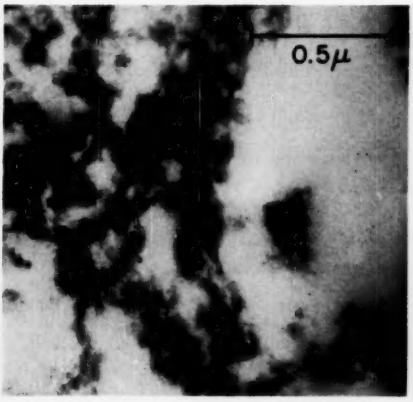


FIG. 10.—Particles in 10mM calcium. Aggregates of dense particles are evident, resembling those in isolated nucleoli.

TABLE 1

INCORPORATION OF OROTIC ACID-6-C^{14} INTO RNA OF SUBNUCLEAR FRACTIONS*

Fraction	Protein (mg)	DNA (mg)	RNA (mg)	Total counts (cts/min)	RNA, specific activity (cts/min/mg)	Composition DNA/RNA
Whole nucleus	1505.2	493.0	48.40	717,000	14,400	10.2
Cytoplasmic fragments and nuclear membranes	57.5	1.59	2.26	5,085	2,250	0.70
Nuclear ribosomes and pH 7.1-extracted proteins	253.6	14.37	24.73	38,200	1,543	0.58
Loose slurry after pH 7.1 Tris extraction	129.1	48.40	3.06	101,000	33,000	15.8
Condensed (hetero-) chromatin	481.5	259.26	7.14	71,855	10,600	36.3
Intermediate	136.8	47.39	3.10	63,230	20,400	15.3
Extended (eu-)chromatin	117.2	44.60	6.23	207,600	33,300	7.1
Particles in 10 mM calcium	65.9	8.95	1.75	106,000	60,600	5.1
Recovery	1237.6	424.44	48.27	592,970		
Per cent	82	86	97	83		

* Orotic acid-6-C^{14} (5.0 mg, 1.86 mC) added to suspension of isolated nuclei in 100 ml of incubation medium (see *Materials and Methods*).

fraction appears as a mixture of condensed (hetero-)chromatin masses and extended (eu-)chromatin fibrils.

The metabolism of DNA, RNA, and protein of the condensed chromatin masses and of the extended chromatin fibrils was studied by means of incubations of the nuclei with C^{14}-labeled precursors. Over 80 per cent of the protein, DNA, RNA, and counts incorporated into new RNA were recovered among the subnuclear fractions (Table 1). Up to 80 per cent of the nuclear DNA is found in the condensed (hetero-)chromatin fraction, but this fraction contains only 17 per cent of the nuclear RNA, and only 14 per cent of the newly synthesized RNA. By contrast, the extended (eu-)chromatin fraction contains the bulk of the newly synthesized RNA, with a sizeable amount also present within the particles in 10 mM calcium (Table 1). Little new RNA is found in the nuclear ribosomes, supporting previous evidence[1] that the chief RNA synthesis in isolated interphase lymphocyte nuclei is of the messenger type. Incubations incorporating thymidine-2-C^{14} into DNA and uridine-2-C^{14} into RNA (Table 2), or lysine-1-C^{14} and alanine-1-C^{14} into protein (Table 3), demonstrate that in each case, condensed (hetero-)chromatin

TABLE 2

INCORPORATION OF C^{14}-LABELED PRECURSORS INTO NUCLEIC ACIDS OF SUBNUCLEAR FRACTIONS

	DNA (mg)	Total counts* (cts/min)	DNA, specific activity (cts/min/mg)	RNA (mg)	Total counts† (cts/min)	RNA, specific activity (cts/min/mg)
Whole nucleus	344.0	228,200	664	40.30	350,000	8,680
Condensed (hetero-) chromatin	109.49	36,530	334	10.385	74,000	7,130
Intermediate	34.20	24,060	704	1.316	10,900	8,280
Extended (eu-)chromatin	35.85	43,950	1224	5.070	90,000	17,750
Particles in 10 mM calcium	5.55	6,020	1085	1.041	24,600	23,650

* Thymidine-2-C^{14} incorporated into DNA, after incubation of nuclei with 0.47 mg (50 μC) of isotope in 100 ml of incubation medium (see *Materials and Methods*).
† Uridine-2-C^{14} incorporated into RNA, after incubation of nuclei with 0.06 mg (7.5 μC) of isotope in 100 ml of incubation medium (see *Materials and Methods*).

TABLE 3

INCORPORATION OF C^{14}-LABELED AMINO ACIDS INTO PROTEINS OF SUBNUCLEAR FRACTIONS

	Protein (mg)	Total counts* (cts/min)	Protein, specific activity (cts/min/mg)	Protein (mg)	Total counts† (cts/min)	Protein, specific activity (cts/min/mg)
Whole nucleus	2118.0	107,200	51	1252.0	283,000	226
Condensed (hetero-)chromatin	500.0	3,670	7	357.2	11,950	34
Intermediate	102.2	4,100	40	97.4	11,950	123
Extended (eu-) chromatin	123.1	7,320	59	99.9	25,100	251
Particles in 10 mM calcium	22.3	1,641	74	22.4	6,744	301

* dl-Alanine-1-C^{14} incorporated into protein after incubation of nuclei with 3.72 mg (160 μC) of isotope in 100 ml of incubation medium (see *Materials and Methods*).

† dl-Lysine-1-C^{14} incorporated into protein after incubation of nuclei with 5.0 mg (95 μC) of isotope in 100 ml of incubation medium (see *Materials and Methods*).

metabolism is $^1/_3$ to $^1/_8$ as active as that of extended (eu-)chromatin. A similar correlation between condensation and inactivity, and between extension and activity has been demonstrated in polytene chromosomes[46] and in lampbrush chromosomes[47, 48] during interphase.

Discussion—The gene-inactivation hypothesis of cell differentiation postulates that as a cell differentiates, those genes not mediating the particular state of differentiation are inactivated by a repression mechanism.[1, 49–52] The present study confirms previous evidence[1,2] that a large fraction of the DNA of interphase calf thymus lymphocytes is metabolically inactive, correlating well with the differentiated state of these cells.[3]

The isolation of repressed and active chromatin from interphase lymphocytes permits studies to elucidate the mechanism of repression in condensed (hetero)-chromatin. If this isolation procedure is gentle enough, the repressed and active chromatin fractions may demonstrate the native *in vivo* association of DNA, histones, nonhistone proteins, RNA, lipids, and other molecular species, permitting an assessment of their role in repression. The histone hypothesis of DNA inactivation is a plausible mechanism for the maintainence of chromatin repression.[1, 2, 25, 53] Current studies in progress[54] suggest that the active, extended (eu-)chromatin fibrils, as isolated by this procedure, have a deficiency of histone for their DNA content, as contrasted to the balance between histone and DNA in the inactive, condensed (hetero-)chromatin masses. A partial account of this study has been presented.[55]

Summary—Repressed and active chromatin can be isolated in mass from interphase calf thymus lymphocytes. Up to 80 per cent of interphase lymphocyte DNA is found in the repressed chromatin fraction. Repressed interphase DNA appears as condensed (hetero-)chromatin, while active interphase DNA appears as extended (eu-)chromatin fibrils. The extended (eu-)chromatin fraction contains the bulk of the rapidly labeled RNA of the nucleus.

The expert technical advice of Dr. Virginia C. Littau and Mr. Donald Orlic regarding the electron microscopy was deeply appreciated.

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*METIN AND METACTOMYOSIN**

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Calcium chloride, if injected into the muscle fiber in minimal quantity, causes an immediate local contraction. Since this initial observation of Heilbrunn and Wiercinski,[1] there has been increasing evidence that Ca^{++} is directly involved in the contraction of actomyosin.[2] This is rather surprising, for the Ca ion, in general, is not known to have an affinity for hydrophilous proteins. Such an affinity could, however, be expected in colloids having numerous negative groups like SO_3, PO_3, or COOH. Since macromolecules carrying such groups may show metachromasia,[3] it appeared worthwhile to seek for a metachromatic macromolecule in muscle.

Fresh, washed or glycerinated muscle, on staining with toluidine blue, turns purple and is thus metachromatic. Microscopically, the fibers appear to be diffusely metachromatic. After disintegrating muscle in a Waring blendor, the myofibrils, separated by low-speed centrifugation, stain metachromatically, whereas the free granules and supernatant are orthochromatic. Thus, the metachromasia seems to be part of the contractile component of muscle.

If freshly minced muscle of a rabbit is repeatedly washed in cold water or extracted in 50 per cent glycerol, it retains the metachromatic property. The metachromatic substance is therefore not easily washed out of muscle. If, however, a watery suspension of the washed muscle is heated to 65°C, then the juice, separated from the particles, shows, on addition of toluidine blue, a metachromatic color while the insoluble residue colors orthochromatically. Hence, denaturation by heating releases a metachromatic substance. On adding the dye in sufficient quantity to the filtrate, a metachromatic precipitate forms; the supernatant remains blue indicating that the metachromatic material is precipitated quantitatively. The precipitate can be separated by centrifugation and the dye dissociated from the precipitated substance (see below). The dye can thus be used for the simple isolation of the metachromatic material.

This material is a protein. It gives a strong biuret reaction, and is precipitated by trichloracetic acid and ammonium sulfate (40 per cent of saturation). It does not precipitate on boiling but does so on addition of acetic acid to the hot solution.

It can also be precipitated, even at a high dilution, isoelectrically around a pH of 5.5–5.7 (addition of NaCl shifts it to a lower pH). The isoelectric precipitation can also be utilized for isolation purposes. Molisch's test was negative.

That the protein is fibrous is indicated by its high viscosity and its double refraction of flow. Addition of 0.1 *M* NaCl greatly decreases the viscosity. Its aqueous solution can be lyophilized.

The protein, which redissolves at higher ionic concentrations, is precipitated by 0.01 *M* $CaCl_2$ or $MgCl_2$. At certain concentrations of NaCl or KCl, Ca has a stronger precipitating action than Mg, a difference which deserves further study. That the dye and the Ca or Mg precipitate one and the same protein, quantitatively, is shown by the fact that the supernatant of the ionic precipitation gives no precipi-

tation with toluidine blue, and vice versa. When a high concentration of monovalent or divalent ions is added to the metachromatic precipitate, it redissolves and becomes orthochromatic.

That this protein may form part of the actomyosin system is suggested by the following. Actomyosin (myosin B) in suspension stains metachromatically. If the actomyosin is heated to 65°C, it denatures and precipitates, whereupon the metachromatic protein goes into solution, giving a metachromatic precipitate on addition of toluidine blue. The metachromatically stained actomyosin still superprecipitates with ATP but at the same time becomes orthochromatic. Actin (G or F, prepared without special precautions for the elimination of tropomyosin) was also found to be metachromatic. Myosin, on the other hand, did not give the metachromatic reaction.

On the basis of its *meta*chromatic property the protein described will be called "*meti*n," while its tertiary complex with actomyosin may be termed "metactomyosin."

Metin has several properties in common with tropomyosin[4] and has also properties common with Amberson's delta protein.[5] Further studies are needed for its characterization.

Extraction Method.—Metin can be prepared from the muscles of a freshly killed rabbit. The skinned and eviscerated rabbit is briefly cooled on ice, its muscles excised and minced on a precooled meat mincer. The mince, say 500 gm, is suspended in 1500 ml of 0°C distilled water, gently shaken for several hr at 0°C, and then filtered through cheesecloth. The extracted muscle is pressed out by hand, suspended once more in 1500 ml of ice-cold water and, after shaking for several hours, is again separated using cheesecloth as before. The muscle is then suspended in 1000 ml of an aqueous 0.05 *M* NaCl solution, and rapidly heated to 65°C while stirring vigorously. The suspension is then cooled on ice, again passed through a cheesecloth, and then centrifuged. It usually gives a limpid supernate. If no NaCl is added, the filtrate is turbid. If the muscle is washed insufficiently and the bivalent ions are not eliminated, the metin extracts poorly or not at all.

Metin can be precipitated from the extract in various ways.

Precipitation by toluidine blue: The pH is adjusted to 7, and a 1 per cent watery solution of toluidine blue is added[6] till the supernate of a small sample is intensely blue and gives no precipitate on addition of more dye. This point is usually reached with the addition of about 0.1 per cent dye. The precipitate is separated by centrifugation, washed with about 200 ml of water, and centrifuged again. Then the precipitate is dissolved in about 100 ml of a 20 per cent acetone containing 0.2–0.4 per cent acetic acid. A homogenizer can be used with advantage. The dilute acetone-acetic acid solution dissolves and dissociates the precipitate into protein and the free blue dye. The latter can be eliminated in various ways. It can be eliminated by adding powdered charcoal (Norit-A) under strong mixing. Since the Norit does not bind the dye quantitatively, it is advantageous to add it only until the fluid is discolored about halfway, then filter through a thin sheet of celite, and repeat the charcoal treatment until practically all the dye is removed. The same can be achieved by dialyzing the solution against the same solvent with liberal amounts of charcoal suspended in the outer fluid.

The metin can be precipitated from the discolored solution by adjusting the pH

to 5–5.7 and centrifuging out the isoelectrically precipitated protein, which readily redissolves on neutralization.

Dr. Eloise E. Clark, to whom our thanks are due, examined such a preparation in the ultracentrifuge and obtained a peak with a Gaussian distribution ($S_{20,w} = 2.85 \times 10^{-13}$; 4.7 mg/ml, 0.1 *M* NaCl). On prolonged spinning, a minute quantity of a slightly faster-sedimenting fraction ($S_{20,w} = 3.6 \times 10^{-13}$) was observed.

The greatest amount of the dye can also be eliminated by precipitating the protein solution from the acetone-acetic acid by the addition of 4 volumes of acetone which brings down the metin in the form of a voluminous precipitate. The solvent is decanted and the voluminous precipitate can be made to collapse by pressing it out with a spatula. The precipitated metin can now be slowly dissolved in neutral or slightly alkaline water. Again, homogenization may be useful. By adding butanol, remnants of the dye may be shaken out of the watery solution.

Our preparation, at this stage, seems to contain traces of nucleotides present as impurity, giving a weak absorption around 270 μM and a weak reaction for phosphate. (A sample studied contained 4.8 mg of protein and 1 μg of P per ml.)

Similar to other metachromatic substances, solutions of metin first color metachromatically on the addition of small quantities of dye and then precipitate only after the dye concentration approaches equimolarity with its binding sites. Approximately one dye molecule is bound by ten amino acids of the metin.

Isoelectric precipitation: Metin can also be precipitated from the primary muscle extract isoelectrically. The NaCl present shifts the isoelectric point to a lower pH. Complete precipitation is obtained in presence of 0.05 *M* NaCl at pH 4.5–5. The precipitate is separated by centrifugation and redissolved by neutralizing the suspension. The protein thus prepared is precipitated quantitatively by toluidine blue, indicating that it is identical with the protein obtained by the dye methods.

Precipitation by bivalent ions: From a salt-free aqueous solution, the metin can also be precipitated quantitatively by 0.01 *M* $CaCl_2$ or $MgCl_2$. This precipitation is prevented by 0.05 M NaCl. If the extraction of muscle was made in the absence of NaCl, the Ca precipitate co-precipitates the substance which has caused the turbidity of the extract. It can be separated from the latter by centrifugation after redissolution. If the solution is still turbid, it can be clarified by adding 0.05 *M* NaCl and 0.01 $MgCl_2$, and centrifuging, the salts being subsequently eliminated by isoelectric precipitation.

* This research was supported by a grant from the National Institutes of Health (GM-10383).

[1] Heilbrunn, L. V., and F. J. Wiercinski, *J. Cellular Comp. Physiol.*, **29**, 15, (1947).

[2] Weber, A., and S. Wincur, *J. Biol. Chem.*, **236**, 3198, (1961).

[3] By "metachromasia" is meant the property of macromolecules to be stained by certain dyes with a color which is different from the orthochromatic color of the dye itself. Toluidine blue, for instance, at high dilution (10^{-6} *M*), at which it is present as a monomer in water, has an absorption at 623 mμ, which corresponds to its blue orthochromatic color. At a higher concentration (10^{-5}) it forms dimers which have a purplish tint and a somewhat weaker absorption at 586. If, for example, polyethylsulphonate of MW 12,900 gm (kindly supplied by Upjohn and Company, Kalamazoo, Michigan) is added, the absorption becomes considerably lower, wider, and shifts strongly to a shorter wavelength (max 526), as also is indicated by the metachromatic purple color. There is no acceptable general theory for this change. Since dimerization shifts the absorption to a somewhat shorter wavelength, it seemed reasonable to suppose that the greater metachromatic shift is due to a further stacking of the dye molecules on the negative groups of the colloid. This theory was revived by F. Bradley, and M. K. Wolf in these PROCEEDINGS, **45**, 844 (1953). How-

ever, it is easy to show that this theory is not generally acceptable. If, for instance, a dilute toluidine blue solution is added gradually to a relatively concentrated solution of a few per cent of polyethylsulfonate, the first trace of dye added, detectable by the naked eye, is already metachromatic. It seems unreasonable to suppose that this minute quantity of dye would form stacks and not distribute itself among the great excess of SO_3 groups. Similarly, if a solution of polyethylsulfonate is gradually added to a solution of toluidine blue, the maximal metachromatic shift is obtained when there is one SO_3 group for every dye molecule. Thus, there being no generally acceptable theory for metachromasia, one may sum up the situation by saying that while the weak interaction between two dye molecules in a dimer causes a small shift and a small decrease in absorbancy, the stronger interaction between the basic dye and strongly negative SO_3, PO_3, or COOH groups causes a greater shift with decrease and broadening of the absorption. Though most proteins contain COOH groups they are not usually metachromatic, the number of COOH groups being low and their electrostatic action being, possibly, also compensated by the NH_2 groups present.

[4] Bailey, K., *Biochem. J.*, **43**, 271, (1948).

[5] White, J. I., H. B. Bensusan, S. Himmelfarb, B. E. Blankenhorn, and W. R. Amberson, *Am. J. Physiol.*, **188**, 212, (1957).

[6] Other metachromatic dyes, like acridine orange, can equally be used.

THE DEPENDENCE OF THE SCHOENFLIES EXTENSION ON AN ACCESSORY PARAMETER (THE TOPOLOGICAL CASE)

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The theorem stated below has been established in detail in a paper published by the authors in 1960[1] and referred to here as (I). The theorem is here restated, with appropriate explanations, because a recent review of this paper in *Mathematical Reviews* refers only to the "differentiable case," whereas in paper (I) the authors explicitly state and prove the theorem both in the "topological case" and the "differentiable case." As a result of this omission in *Mathematical Reviews*, mathematicians have started work on the problem in the "topological case" under the assumption that (I) covers *only* the differentiable case.

The parameter p in the problem in the topological case has for domain an arbitrary *paracompact space* Γ.

Let E be a Euclidean n-space $n > 1$ of points x with rectangular coordinates $(x_1, \ldots, x_n)$. Let S be an $(n-1)$-sphere in E, and B the closed n-ball bounded by S.

The Product Space $E \times \Gamma$.—Let $\mathbf{x}$ and p be points in E and Γ, respectively, and X a subset of $E \times \Gamma$. One introduces the projections

$$pr_1 : E \times \Gamma \to E; \qquad pr_1(x,p) = x$$
$$pr_2 : E \times \Gamma \to \Gamma; \qquad pr_2(x,p) = p.$$

For p fixed in Γ, the *p-section* of X is by definition the set

$$X^p = \{x \in E | (x,p) \in X\}.$$

If $(x,p) \to F(x,p)$ is a mapping of X into $E \times \Gamma$ set,

$$pr_1 F(x,p) = F_1(x,p), \qquad pr_2 F(x,p) = F_2(x,p).$$

Thus, $(x,p) \to F_1(x,p)$ is a mapping of X into E, and $(x,p) \to F_2(x,p)$ is a mapping of X into Γ. The mapping F will be termed *p-invariant* if for each $(x,p) \in X$, $F_2(x,p) = p$. For $(x,p) \in X$ one sets

$$F_1(x,p) = F^p(x),$$

and for fixed $p \in pr_2X$ terms the mapping $x \to F^p(x)$ of X^p into E the p-section of F.

The Data.—Let L be an open neighborhood of $S \times \Gamma$ relative to $E \times \Gamma$, and let there be given a p-invariant homeomorphism

$$\Phi: L \to E \times \Gamma; \qquad (x,p) \to \Phi(x,p)$$

such that for each $p \in \Gamma$, Φ^p maps points of L^p which are interior (exterior) to S in E into points which are interior (exterior) to the topological $(n-1)$-sphere $\Phi^p(S)$ in E. Set

$$L_e = L - (B \times \Gamma).$$

THEOREM. *Corresponding to Φ, L, and Γ, conditioned as above, and to any sufficiently small open neighborhood $L_* \subset L$ of $S \times \Gamma$ relative to $E \times \Gamma$, there exists a p-invariant homeomorphism*

$$\Lambda: L \cup (B \times \Gamma) \to E \times \Gamma$$

which extends $\Phi | (L_e \cup L_*)$.

For fixed $p \in \Gamma$, Λ^p is a solution of the classical Schoenflies problem in which $\Phi^p: L^p \to E$ is the given homeomorphism. Moreover, Λ^p varies continuously with p, since Λ is continuous. The problem with parameter is made difficult by the fact that the domain L^p of definition of Φ^p in E varies with p. In fact, it is not excluded that the subset

$$\bigcap_p L^p \quad (p \in \Gamma)$$

of E fails to be a neighborhood of S in E.

A solution of the "problem with parameter" is made possible by the *explicit* nature of the authors' earlier solution of the classical Schoenflies problem without parameter.[2] Formulas in reference 2 in the classical problem which give an extension of a homeomorphism φ explicitly involve a restriction of φ. These formulas are of such a nature that they apply whether φ is a *homeomorphism*, a *diffeomorphism*, or even an *analytic diffeomorphism*.[3] They apply in the case at hand when φ is replaced by a mapping Φ which depends upon a parameter p, provided a preliminary transformation of the Γ-problem into a "*uniform*" Γ-problem is made (cf. ref. 1).

In the differentiable case Γ is assumed to be a connected differentiable r-manifold, $r > 0$ of class C^m, $0 < m \leq \infty$, with a countable base. For statement of the theorem in the differentiable case see (I) or *Mathematical Reviews*.

[1] Huebsch, W., and M. Morse, "The dependence of the Schoenflies extension on an accessory parameter," *Jour. d'Analyse Math.*, **8**, 209–271 (1960–61).

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*ON A "MONOTONICITY" METHOD FOR THE SOLUTION OF NONLINEAR EQUATIONS IN BANACH SPACES**

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Communicated by John W. Tukey, October 9, 1963

1. *Introduction.*—Unless otherwise explicitly stated, X is a reflexive Banach space and Y its "conjugate" or "adjoint" space. For $x \in X$, $y \in Y$, the symbol $\langle x, y \rangle$ means the value assumed by y at x if the conjugacy is with respect to real numbers, and is the real part of this value if the conjugacy is with respect to complex numbers. The zero-vector of a linear space is called θ; we shall never be in doubt which space it belongs to.

In harmony with definitions introduced in earlier papers for Hilbert spaces only,[3, 5, 6] we say that a pair (x_1, y_1), (x_2, y_2) in $X \times Y$ is *M-related* provided $\langle x_1 - x_2, y_1 - y_2 \rangle \geq 0$. A set $G \subset (X \times Y)$ is *totally M-related* provided each pair of points of G is M-related, and is called *maximal* if it is not properly contained in a totally-M-related set.

For $B \subset X$, a function $f: B \to Y$ is called *monotonic* if its graph is a totally-M-related set, i.e., if $\langle x_1 - x_2, f(x_1) - f(x_2) \rangle \geq 0$. Extending slightly a definition of F. E. Browder,[1] we call f *hemicontinuous at* $x_0 \in B$ provided, for every half-open (real) line-segment T in X with endpoint x_0, f is continuous at x_0 considered as a map from $T \cap B$ into Y, where Y is taken with the weak topology (induced by X). All topological terms refer to the "strong" or "norm" topology unless the contrary is explicitly stated. The symbol $K(B)$ denotes the *closed* convex hull of B; we remind the reader of the well-known theorem which asserts that this set is weakly closed.

The set B will be said to *surround* x_0 provided, for each (real) line $T(x_0, z) = \{x: x = x_0 + tz, t \text{ real}\}$ $(z \in X, z \neq \theta)$ containing x_0, B contains one point of T on each side of x_0, and will be said to surround x_0 *densely* provided for each T, on each side of x_0, it contains points of T arbitrarily close to x_0. In neither of these two definitions does B need to contain x_0.

The object of this note is to generalize theorems of the author,[3, 5] and F. E. Browder,[1] stated for Hilbert spaces only, on the existence of solutions of $f(x) = \theta$. At the same time, Theorem 1 of this paper attempts to generalize Theorem 7 of Vainberg and Katchurovskii.[7] (The theorem of ref. 7 is essentially the special case in which f is the gradient of a real scalar functional. However, in ref. 7 the function f is required to be everywhere-defined in X, and an asymptotic property as $\|x\| \to \infty$ replaces our condition (1) below.)

It is anticipated that Theorem 1 will find application to proofs of existence of solutions of nonlinear integral equations, with L^p-spaces and Orlicz spaces as the context (see refs. 3, 4, and 7 for further information). It is also felt that the theorem will facilitate the development of a theory of nonlinear partial differential equations, with Sobolev spaces of L^p-type as the context.

During the drafting of this paper, Browder[2] has communicated to the author an independently proved generalization of Theorem 7 of reference 7 in a somewhat different direction, while requiring separability of the Banach space X, and carried

out part of the program of application to partial differential equations.

The main theorem is:

THEOREM 1. *Let $D \subset X$ be bounded and surround θ; let $B \subset X$ contain $K(D)$ and surround every point of $K(D)$ densely. Let $f:B \to Y$ be monotonic and hemicontinuous at every point of $K(D)$, and suppose*

$$x \in D \text{ implies } \langle x, f(x)\rangle \geq 0. \tag{1}$$

Then there exists $x \in K(D)$ such that $f(x) = \theta$.

2. *Remark.*—For the sake of concreteness, Lemma 1 and Theorem 1 were stated and will be proved for a reflexive Banach space X with conjugate space Y. However, we point out

THEOREM 2. *The statement of Theorem 1 holds if Y is any Banach space (reflexive or nonreflexive), X is its conjugate space, and f maps $B \subset X$ into Y, provided that the weak-star topology is used in the definitions of $K(D)$, weak closure, and hemicontinuity.*

The proof is the same, with the obvious replacements.

In a typical application, D would be the boundary of a large ball with center θ, and B would be an open sphere containing D.

3. *Lemmas.*—Lemma 1 is a mild generalization of Lemma 1 of reference 1, which is stated for Hilbert spaces, and is in turn a generalization of part of a lemma of the writer (Theorem 4 of ref. 5).

LEMMA 1. *Let $B \subset X$ contain x_0 and surround x_0 densely; let $f:B \to Y$ be hemicontinuous at x_0. Let $(x_0, y_0) \in (B \times Y)$ be M-related to every point of the graph of f. Then $y_0 = f(x_0)$.*

Proof: Suppose the conclusion false: $\|f(x_0) - y_0\| > 0$. Choose a nonzero vector $z \in X$ such that

$$\langle z, f(x_0) - y_0\rangle \geq \|z\| \cdot \|f(x_0) - y_0\|/2. \tag{2}$$

(This choice is possible by the definition of the norm in Y and, if necessary, multiplication of z by an appropriate scalar of modulus 1.) By hemicontinuity of f at x_0 and the fact that B surrounds x_0 densely, we can choose a real positive t such that $(x_0 - tz) \in B$ and

$$|\langle z, f(x_0 - tz) - f(x_0)\rangle| \leq \|z\| \cdot \|f(x_0) - y_0\|/3. \tag{3}$$

Now, since (x_0, y_0) is M-related to every point of the graph of f, we have

$$\langle (x_0 - tz) - x_0, f(x_0 - tz) - y_0\rangle \geq 0.$$

Canceling x_0, dropping the (real, positive) t, adding and subtracting $f(x_0)$ in the right hand entry and rearranging, we obtain

$$\langle -z, f(x_0 - tz) - f(x_0)\rangle \geq \langle z, f(x_0) - y_0\rangle.$$

Taking the absolute value on the left and employing (2) on the right, we see

$$|\langle z, f(x_0 - tz) - f(x_0)\rangle| \geq \|z\| \cdot \|f(x_0) - y_0\|/2$$

in contradiction with (3), since $\|z\|$ and $\|f(x_0) - y_0\|$ are nonzero.

The next lemma and its proof are modifications of Appendix II of reference 3.

LEMMA 2. *Theorem 1 holds if X is finite dimensional.*

Proof: (Notice that, in this case, the statement of the theorem need not involve

the Banach space norm!) Choose a basis in X, coordinatize, and introduce an inner product in the usual way; thus X becomes a Hilbert space, and we make the canonical identification of Y with X. In the rest of this proof, $\|x\|$ refers to the Hilbert space norm.

For each positive integer n, consider the graph G_n of the function $nf(x)$. Now, G_n is a totally-M-related set; by the usual Zorn's Lemma argument, it can be extended to a maximal totally-M-related set G_n'. Theorem 3 of reference 5 asserts that the map $(x, y) \to x + y$ carries G_n' *onto* X. Thus, there exists $(x_n, y_n) \in G_n'$ such that $x_n + y_n = \theta$. Let us show that (for all n) $x_n \in K(D)$. Assume the contrary.

Let z_n be a point of D such that $x_n = \lambda_n z_n$ for real $\lambda_n > 1$; this is possible because D surrounds θ and $x_n \notin K(D)$. Now, $x_n + y_n = \theta$, so forming the inner product with x_n, we have $\|x_n\|^2 + \langle x_n, y_n\rangle = 0$; neglecting the easy case $x_n = \theta$, we see $\langle x_n, y_n\rangle < 0$, and hence, $\langle z_n, y_n\rangle < 0$. But $\langle z_n, nf(z_n)\rangle \geq 0$, and thus,

$$(\lambda_n - 1)\langle z_n, y_n\rangle < (\lambda_n - 1)\langle z_n, nf(z_n)\rangle$$

which can easily be rearranged to

$$\langle x_n - z_n, y_n - nf(z_n)\rangle < 0,$$

showing that (x_n, y_n) is not M-related to $(z_n, nf(z_n))$, in contradiction with $(x_n, y_n) \in G_n'$. Hence, $x_n \in K(D)$.

It follows by Lemma 1 that $y_n = nf(x_n)$, and thus,

$$x_n/n + f(x_n) = \theta. \tag{4}$$

Now, since E is finite-dimensional, $K(D)$ is compact; choose a convergent subsequence $\{x_{ni}\}$ with limit x. We shall use Lemma 1 to show $f(x) = \theta$. The set B surrounds x densely; let x_0 be any element of B. Then by monotonicity of f, $\langle x_0 - x_{ni}, f(x_0) - f(x_{ni})\rangle \geq 0$; substituting from (4), we obtain

$$\langle x_0 - x_{ni}, f(x_0)\rangle + \langle x_0, x_{ni}\rangle/n_i - \|x_{ni}\|^2/n_i \leq 0.$$

in the limit as $i \to \infty$, the second and third terms go to zero, we obtain $\langle x_0 - x, f(x_0) - \theta\rangle \geq 0$, and the conclusion follows by Lemma 1.

4. *Proof of Theorem 1.*—We now proceed to the proof of Theorem 1 in the general case. We wish to show that there exists $x \in K(D)$ with $f(x) = \theta$. By the fact that B contains and surrounds densely every point of $K(D)$, and Lemma 1, it suffices to show that the set

$$K(D) \cap [\cap_{x_0 \in B}\{x: \langle x_0 - x, f(x_0) - \theta\rangle \geq 0\}]$$

is nonempty. We shall show (equivalently) that the set

$$\cap_{x_0 \in B}\{x: \langle x_0 - x, f(x_0)\rangle \geq 0, x \in K(D)\}$$

is nonempty. Now, the sets of this intersection are all weakly closed subsets of the weakly compact set $K(D)$, so that it suffices to show that the intersection of any finite subcollection of these sets is nonempty.

We shall show that, for $x_1, \ldots, x_m$ in B, there exists $x \in K(D)$ with

$$\langle x_i - x, f(x_i)\rangle \geq 0 \quad (i = 1, \ldots, m). \tag{5}$$

Let $E \subset X$ be the finite-dimensional subspace spanned by $x_1, \ldots, x_m$. Now, for $y \in Y$ and variable $x \in E$, $y(x)$ is a continuous linear functional on E which we denote by y'. Thus, there is defined in a natural way a function $f'(x)$ with domain $(E \cap B)$ and range in E^*, the conjugate space of E. The domain contains and surrounds densely every point of $E \cap K(D)$, hence every point of $K(E \cap D)$. It is easy to show that f' is monotonic and hemicontinuous. The set $(E \cap D)$ surrounds the origin in E, and $x \in (E \cap D)$ implies $\langle x, f'(x)\rangle \geq 0$. All the hypotheses of Lemma 2 are satisfied, so there exists $x \in K(E \cap D)$ with $f'(x) = \theta$. Of course, it does not follow that $f(x) = \theta$; however, by the monotonicity of f', $\langle x_i - x, f'(x_i) - \theta\rangle \geq 0$ for $i = 1, \ldots, m$, and from this (5) follows, since all the $(x_i - x)$ are elements of E. The proof is complete.

The author is indebted to R. G. Douglas for several helpful conversations, to F. E. Browder for prepublication copies of his papers, and to C. L. Dolph for encouragement.

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*SPECTRAL OPERATORS IN A DIRECT SUM OF HILBERT SPACES**

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The purpose of this communication is to outline methods which yield a fairly complete spectral analysis for operators in a certain noncommutative B^*-algebra of operators in the direct sum $\mathfrak{H}^n = \mathfrak{H} + \ldots + \mathfrak{H}$ of a Hilbert space $\mathfrak{H}$ with itself n times. Some of the results described herein are intimately related to the elegant work of S. R. Foguel[1] in his study of the algebra $\mathfrak{A}$ of operators commuting with a normal operator of finite multiplicity.

Let $\sum$ be a σ-field of subsets of a set $\mathfrak{S}$ and $e(\cdot)$ a countably additive self adjoint spectral measure defined on $\sum$ whose values are projection operators in $\mathfrak{H}$. Let $\mathfrak{B}$ be the algebra of all operators b in $\mathfrak{H}$ having the form $b = \int_{\mathfrak{S}} \hat{b}(\mathfrak{f}) e(d\mathfrak{f})$ for some e-essentially bounded $\sum$-measurable function $\hat{b}$ on $\mathfrak{S}$. This correspondence $b \leftrightarrow \hat{b}$ is known[2] to be an isometric $*$-isomorphism between the algebra $\mathfrak{B}$ and the algebra $eB(\mathfrak{S})$ of e-essentially bounded functions on $\mathfrak{S}$. Now consider the algebra of

mappings B: $[x_1, \ldots, x_n] \to [y_1, \ldots, y_n]$ in $\mathfrak{H}^n$ having the form $y_i = \sum_{k=1}^n b_{ik}x_k$ where b_{ij} is in $\mathfrak{B}$. This is a noncommutative B^*-algebra $\mathfrak{B}^n$ of operators in $\mathfrak{H}^n$ which is isometrically $*$-isomorphic with the algebra $eB^n(\mathfrak{S})$ of all $\sum$-measurable e-essentially bounded maps $\mathfrak{f} \to \hat{B}(\mathfrak{f})$ of $\mathfrak{S}$ into the algebra $B(E^n)$ of bounded linear operators in n-dimensional unitary space E^n. The isomorphism is given by the integral $B = \int_{\mathfrak{S}} \hat{B}(\mathfrak{f})e(d\mathfrak{f})$. It follows that the spectrum of B consists of those λ for which e-ess $\sup_{\mathfrak{f} \in \mathfrak{S}} |\det(\lambda I - \hat{B}(\mathfrak{f}))|^{-1} < \infty$. Stated otherwise,

$$\sigma(B) = \bigcap_{e(\delta) = I} \overline{\bigcup_{\mathfrak{S} \in \delta} \sigma(\hat{B}(\mathfrak{f}))}.$$

Now, for a fixed $\mathfrak{f}$ in $\mathfrak{S}$, the operator $\hat{B}(\mathfrak{f})$ in E^n, being a spectral operator, has a resolution of the identity whose value on the Borel set σ of complex numbers we will denote by $E(\sigma;\hat{B}(\mathfrak{f}))$. It may be proved that the condition

$$\sup_{\sigma \in \mathfrak{B}} \; e\text{-ess} \sup_{\mathfrak{f} \in \mathfrak{S}} \; |E(\sigma;\hat{B}(\mathfrak{f}))| < \infty, \tag{1}$$

where $\mathfrak{B}$ is the Borel field in the complex plane, is equivalent to the condition

$$e\text{-ess} \sup_{\mathfrak{f} \in \mathfrak{S}} \; \sup_{\sigma \in \mathfrak{B}} |E(\sigma;\hat{B}(\mathfrak{f}))| < \infty. \tag{1'}$$

This condition implies the following Fubini type formula. Let φ be a bounded Borel scalar function on the spectrum $\sigma(B)$. Then the integral $\int_{\sigma(B)} \varphi(\lambda)E(d\lambda;\hat{B}(\mathfrak{f}))$ is an e-essentially bounded $\sum$-measurable function of $\mathfrak{f}$, the integral $\int_{\mathfrak{S}} E(\sigma;\hat{B}(\mathfrak{f}))$ $e(d\mathfrak{f})$ is a bounded countably additive spectral measure in $\mathfrak{H}^n$, and

$$\int_{\mathfrak{S}} [\int_{\sigma(B)} \varphi(\lambda)E(d\lambda;\hat{B}(\mathfrak{f}))]e(d\mathfrak{f}) = \int_{\sigma(B)} \varphi(\lambda)[\int_{\mathfrak{S}} E(d\lambda;\hat{B}(\mathfrak{f}))e(d\mathfrak{f})]. \tag{2}$$

From this formula it may be proved that the operator B in $\mathfrak{H}^n$ is a spectral operator if and only if the condition (1) holds, and that when it is a spectral operator, it is of type $n - 1$, i.e., the nth power of its radical vanishes. The resolution of the identity for B is the integral

$$E(\sigma;B) = \int_{\mathfrak{S}} E(\sigma;\hat{B}(\mathfrak{f}))e(d\mathfrak{f}).$$

From this result, it is clear that every operator in $\mathfrak{B}^n$ is the strong limit of a sequence of spectral operators, a result proved by Foguel for the algebra $\mathfrak{A}$ mentioned earlier.

The projections $E(\sigma;\hat{B}(\mathfrak{f}))$ may be calculated as polynomials in $\hat{B}(\mathfrak{f})$ by using interpolating polynomials,[3] and an examination of the form of these polynomials shows that if, for e-almost all $\mathfrak{f}$ in $\mathfrak{S}$ and some constant K, we have $\sup|\lambda - \mu|^{-1} < K$, where the supremum is taken over all λ, μ of distinct eigenvalues of $\hat{B}(\mathfrak{f})$, then B is a spectral operator of type $n - 1$. However, this condition is not necessary.

Let the complex number system be ordered by defining $w \; \mathfrak{r} \; z$ to mean that $|w| \leqq |z|$ and if $|w| = |z|$, then $\arg w < \arg z$. Let $\mathfrak{S}_i$ be the set of all $\mathfrak{f}$ in $\mathfrak{S}$ for which the spectrum $\sigma(\hat{B}(\mathfrak{f}))$ consists of i distinct points, and let $\hat{\lambda}_{ij}(\mathfrak{f})$, $j = 1, \ldots, i$ be these distinct characteristic numbers arranged so that $\hat{\lambda}_{i1}(\mathfrak{f}) \; \mathfrak{r} \; \ldots \; \mathfrak{r}\hat{\lambda}_{ii}(\mathfrak{f})$. The sets $\mathfrak{S}_i$ are disjoint sets in $\sum$, whose union is $\mathfrak{S}$, and the function $\hat{\lambda}_{ij}(\cdot)$ is a $\sum$-measurable function on $\mathfrak{S}_i$. If $E(\hat{\lambda}_{ij}(\mathfrak{f});\hat{B}(\mathfrak{f}))$ is the value of the resolution of the identity of $\hat{B}(\mathfrak{f})$ evaluated on the set consisting of the single point $\hat{\lambda}_{ij}(\mathfrak{f})$, then the condition (1) is also equivalent to the condition

$$e\text{-ess} \sup_{\mathfrak{f} \in \mathfrak{S}_i} |E(\hat{\lambda}_{ij}(\mathfrak{f});\hat{B}(\mathfrak{f}))| < \infty, \; 1 \leqq j \leqq i \leqq n. \tag{1''}$$

In terms of these projections, the operational calculus for the spectral operator B may be put in a form reminiscent of that for a finite matrix. By way of illustration, let S be the scalar part of B and let φ be a bounded Borel scalar function on $\sigma(B) = \sigma(S)$. Then the operator $\varphi(S)$ in $\mathfrak{H}^n$ is given by the formula $\varphi(S) = \sum_{i=1}^{n} \sum_{j=1}^{i} \varphi(\lambda_{ij})E_{ij}$, where $E_{ij} = \int_{\mathfrak{S}_i} E(\hat{\lambda}_{ij}(\mathfrak{f}); \hat{B}(\mathfrak{f}))e(d\mathfrak{f})$ is a projection in $\mathfrak{H}^n$ and the elements λ_{ij}, $1 \leqq j \leqq i \leqq n$, are operators in $\mathfrak{H}$ defined by the equations $\lambda_{ij} = \int_{\mathfrak{S}_i} \hat{\lambda}_{ij}(\mathfrak{f})e(d\mathfrak{f})$.

The above remarks show that the spectral analysis of such operators B reduces to the algebraic problem of solving a polynomial of degree n. Among the familiar operations of mathematical analysis which come under the preceding discussion, that of convolutions by L_1 functions is perhaps the most interesting. By taking the Hilbert space $\mathfrak{H}$ to be $L_2(-\infty, \infty)$, and by taking the operators b_{ij} in $L_2(-\infty, \infty)$ to be operators of the form

$$b_{ij}f = \alpha_{ij}f + g_{ij}*f, \quad f \in L_2(-\infty, \infty),$$

where α_{ij} is a complex number, g_{ij} is a function in $L_1(-\infty, \infty)$, and

$$g_{ij}*f = \int_{-\infty}^{\infty} g_{ij}(s - t)f(t)dt,$$

it is seen that a complete spectral analysis of the operator $B = (b_{ij})$ in $\mathfrak{H}^n$ reduces to the examination of an $n \times n$ matrix whose elements are given in terms of the Fourier transforms of the functions g_{ij}.

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*BACTERIOPHAGE-INDUCED MUTATION IN ESCHERICHIA COLI**

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Many hereditary traits of bacteria are determined by a special class of genetic elements termed *episomes*.[1–4] Episomes usually determine nonessential characters not ordinarily represented in the bacterial gene pool. Some episomic elements, e.g., transducing phages and the sex factor of *Escherichia coli* strain K12, are instrumental in providing means for the intercellular transfer of bacterial genes. It has been postulated that episomes may also regulate the biosynthetic activities of bacteria at the genetic level by stimulating, inhibiting, or otherwise modifying the phenotypic expression of specific genes.[1, 5] Recent discoveries[5–7] indicate that episomes can in fact stimulate the activity of bacterial genes under special conditions, but total suppression of normal genetic functions by an episome has not been observed in Eubacteria.

This report describes a novel temperate bacteriophage, designated phage Mu1,

that causes severe genetic modifications in its natural host, *E. coli* K12. The prophage of Mu1 abolishes normal phenotypic expression of many host genes located at (or adjacent to) the chromosomal sites of phage integration. The over-all effect of Mu1 on bacteria is *superficially* indistinguishable from that of a mutagen. Hence, the phenomenon described here will be referred to as phage-induced mutation.

Materials and Methods.—Bacteria and phages: Strain AB1172, given to us by N. B. Groman, is a prototrophic derivative of *E. coli* K12 that does not carry lambda prophage. Strain Sh of *Shigella dysenteriae* and phage P1*kc* were kindly provided by S. E. Luria. Phage Mu1 was isolated from a naturally occurring lysogen of *E. coli*. Phage Mu1*c* is a clear plaque mutant of Mu1 that has a reduced capacity to lysogenize strain AB1172.

Media: A half-strength preparation of medium 56 described by Monod *et al.*[8] was used in all minimal media (MM). It was supplemented with glucose or other carbon sources at 2 mg/ml; with required amino acids, purines, and pyrimidines at predetermined optimal concentrations varying from 20 to 140 μg/ml; with vitamins at 2 μg/ml; and with 2% agar in solid media. Partially enriched minimal medium (EMM) for the detection of nutritional auxotrophs was MM containing 200 μg/ml of Difco nutrient broth powder. A tryptone-yeast extract medium[9] (L broth) was used for cultivating bacteria and as diluent for phages. Agar plates (PL agar) for phage assays and preparation of lysates contained L broth solidified with 1% agar and supplemented with the following adsorption cofactors: 2.5×10^{-3} *M* $CaCl_2$, 10^{-3} *M* $MgSO_4$, and 5×10^{-4} *M* L-tryptophan. Soft agar for pouring top layers was L broth containing 0.6% agar.

Phage assays and high titer phage lysates were prepared by the agar layer method outlined by Adams.[10] Bacteria were usually scored for Mu1 lysogeny by replica plating[11] inocula from master plates onto two successive PL plates, one containing 10^8 sensitive indicator bacteria embedded in a soft agar layer, and the other containing 10^9 Mu1*c* phages spread uniformly on the agar surface. The first plate differentiated lysogens from nonlysogens on the basis of phage liberation, whereas the second plate did so on the basis of immunity.

Auxotrophic mutants were distinguished from prototrophs by their minute colony size on EMM plates. Small colonies were transferred to L plates with the aid of a fine needle and a dissecting microscope. After incubation, mutants on the L plates were characterized by replica plating onto MM containing various growth factors.

Tests for reversion to prototrophy were made by plating approximately 5×10^7 washed cells on EMM and incubating at 37°C for 5–6 days. Sufficient plates were prepared in each test to give final populations of 10^9 to 10^{10} bacteria.

Results.—Source and characterization of phage Mu1: Phage Mu1 was discovered by chance during tests for lysogeny in an *E. coli* strain that had been exposed to phage P1. A chloroform-treated broth culture of this strain was seeded onto indicator plates of *Sh. dysenteriae* to check for free P1 phage. Surprisingly, none of the plaques that appeared after incubation had the morphological characteristics of P1. Phages taken from one of the plaques plated with equal efficiency on both P1 lysogens and nonlysogens; moreover, antiserum prepared against P1 did not inactivate this phage. The original bacterial strain and its immediate ancestor, which had not been exposed to P1, were immune to the new phage, but uniform sensitivity was the rule for all other K12 stocks. Therefore, phage Mu1 was not related to P1, and its unexpected appearance in our strains was attributed to chance contamination.

Phage Mu1 was also unrelated to phage lambda, the only species normally present in strain K12, for no serological cross-reaction, no cross-immunity, and no cross-resistance could be demonstrated between the two phages. Similarly, there was no evidence of serological relationship between Mu1 and temperate coliphage P2.

Phage Mu1 plated with equal efficiency on *Sh. dysenteriae* and on the three mating types (F^+, Hfr, F^-) of *E. coli* K12. No plaques appeared when 5×10^9 particles

were plated on *E. coli* strains B, W, and S, or on *Salmonella typhimurium* strain LT-2. On the basis of its efficient sedimentation in 1 hr at 20,000 × *g*, the particle weight of Mu1 was estimated to be about the same as that of lambda. A detailed physical and chemical characterization of Mu1 is now in progress.

Mutations in cultures infected with Mu1: Phage-induced mutations were first noticed in experiments to prepare lysogenic derivatives of multiply auxotrophic K12 stocks. Droplets containing approximately 10^8 phage particles were placed on PL agar plates containing 2×10^8 bacteria spread over the agar surface. Confluent growth of presumptive lysogenic survivors became visible in the lysed areas after 8 hr of incubation at 37°C. This growth was emulsified in sterile buffer and streaked on L plates to obtain isolated colonies. Colonies were then scored for both lysogeny and for continued presence of the original auxotrophy markers. Of 160 colonies tested, all were lysogenic for Mu1, all retained the original nutritional requirements, but three required an additional amino acid for growth on minimal medium. The original uninfected stock was checked for contaminants or high levels of spontaneous mutants, but neither was found. Therefore, it seemed as if lysogenization was instrumental in producing the three new mutants.

When the preceding experiment was repeated on a larger scale, lysogenic survivors consistently contained 1–3 per cent of auxotrophic mutants (Table 1). As shown in column 5 of Table 1, mutants within a single population were often phenotypically dissimilar. This suggested that many independent mutational events occurred in the infected cultures. Phage propagated on strains other than AB1172, namely, *Sh. dysenteriae* and a K12 multiple auxotroph, produced mutants in AB1172 at frequencies similar to those given in Table 1.

The nutritional requirements of phage-induced mutants were as varied as those normally encountered in mutagen-treated bacteria. The 414 auxotrophs examined to date all represented one-step mutations in a minimum of 34 different loci. The following list identifies the 34 phenotypes and the frequency of their occurrence as independent mutations: adenine (24), adenine or hypoxanthine (11), adenine + thiamine (20), arginine (12), cysteine (8), cysteine or thiosulfate (9), cysteine or sulfite (15), glycine (4), glutamic acid (2), guanine (4), histidine (17), homocystine

TABLE 1

FREQUENCY OF MUTANTS IN CULTURES INFECTED WITH PHAGE MU1*

Culture	No. of colonies scored	No. of auxotrophs	Frequency (%)	No. of phenotypes
1	390	11	2.6	6
2	700	15	2.1	8
3	330	5	1.5	4
4	580	13	2.2	8
5	280	7	2.5	7
6	500	10	2.0	8
7	490	6	1.2	5
8	740	17	2.3	11
9	510	8	1.6	7
10	1160	23	2.0	16
11	340	10	2.9	8
12	370	10	2.7	5
13	560	10	1.8	7
Control	2400	0	...	...

* Exponential phase cells of AB1172 were spread on PL plates and exposed, as described in the text, to phage Mu1 prepared on strain AB1172. Samples from 13 separate zones of lysis were assayed for auxotrophic mutants by spreading cells on EMM plates; samples taken from uninfected areas served as controls. Presumptive mutants (minute colony formers on EMM) were positively identified by determining their specific growth factor requirements.

(4), indole (7), isoleucine (3), isoleucine + valine (8), leucine (16), lysine (4), lysine + methionine (8), methionine (19), methionine or cobalamin (10), multiple aromatic amino acids (13), nicotinic acid (1), ornithine (4), orotic acid (23), pantothenic acid (2), phenylalanine (9), proline (32), serine or glycine (25), shikimic acid (5), threonine (39), tryptophan (20), tyrosine (7), uracil (23), uracil + arginine (6).

Syntrophy tests and growth responses to precursor metabolites indicated that phage-induced mutants were probably all deficient for single biosynthetic enzymes. Only one mutant, however, has been studied directly at the enzymological level. Enzyme assays showed that derepressed cells of a uracil-requiring mutant lacked the enzyme orotidylic acid pyrophosphorylase but retained normal levels of other enzymes in the uracil pathway.[12] In addition to auxotrophs, mutants resistant to 0.002 *M* sodium azide, mutants unable to use lactose as sole carbon source, and mutants resistant to phages T6, T4, T5, and T1 also appeared frequently in Mu1-infected cultures. Several working hypotheses were developed to account for the occurrence of these diverse mutations. These will be discussed in the following sections.

(*a*) *Selection:* The low variance in mutant frequencies noted in 13 different populations derived from the same initial culture (cf. Table 1), plus the observation that these populations often contained phenotypically identical mutants, suggested selective lysogenization of spontaneous mutants as a possible mechanism. This model predicted: (1) that treatments to reduce the number of spontaneous mutants in cultures prior to infection would also reduce the yield of mutants after infection; (2) that auxotrophic mutants arising in populations of multinucleate cells would be selected only after gaining phenotypic expression and therefore would produce only pure clones of selected mutants; (3) that the mutation *rate* of a particular gene would be the same in both infected and uninfected cultures.

The first prediction was tested by serially culturing strain AB1172 three times from small inocula in minimal medium. When this culture, which presumably contained few auxotrophs, was exposed to Mu1 in the manner previously described, mutants were recovered from it at about the same frequency as in the previous experiments (*ca.* 2 per cent).

The second prediction was tested by recovering the progeny of single infected cells as isolated clones. Small numbers of multinucleate bacteria were mixed with 10^8 to 10^9 phage particles on PL plates and incubated until survivors produced visible colonies.[13] Individual colonies were then scored for the presence of wild-type and auxotrophic bacteria. As shown in Table 2, 43 per cent of the colonies tested were mixed clones containing both wild-type and mutant progeny. Colonies composed exclusively of mutants were not found. Of the 46 mixed clones found in two experiments, 27 contained only one kind of auxotroph, 12 contained two kinds, 6 contained three kinds, and 1 contained four kinds. The proportion of auxotrophs per clone was only 1–2 per cent for 34 of the colonies; the proportion in the 12 remaining colonies was much greater, between 10–65 per cent. This implied early segregation of mutant characters in the latter clones and, by inference, early induction of mutations in the infected cells.

The third prediction was tested by measuring the rate of mutation to phage T1 resistance ($T1^r$) in a culture infected with phage Mu1. Mutation rates were determined by the agar plate method of Demerec[14] and Beale,[15] with the following

TABLE 2

FREQUENCY OF MUTANTS IN SINGLE LYSOGENIC CLONES*

Experiment no.	Survival (%)	Total no. of colonies tested	No. of Colonies Containing Mutants as: Pure clones	Mixed clones
1	15	56	0	23
Control	100	18	0	0
2	7	51	0	23
Control	100	25	0	0

* Exponential phase cells (5×10^3) of AB1172 plus 2×10^8 phages were spread together on PL agar and incubated 16 hr at 37°C. Phage was omitted in control platings. Whole colonies were removed, dispersed in buffer, and streaked on EMM plates to give 300–600 subclones. Minute colony formers were identified as auxotrophic mutants by determining their specific growth factor requirements.

modification: instead of spraying test plates with phage T1 aerosols, T1 was transferred to the dishes by replica plating from velvet cloth impregnated with sufficient phage particles to cause complete lysis of sensitive bacteria. The latter technique was more convenient than the former, but less accurate because some $T1^r$ cells that had not yet formed clones on the plates adhered to the velvet, thus permanently escaping detection. Regardless of this limitation, the spontaneous mutation rate of 1.8×10^{-8} per bacterium per division cycle found in strain AB1172 (Table 3) agreed well with previous determinations in *E. coli* strain B/r.[14–16] The mutation rate of bacteria that survived infection was 63 times greater than the spontaneous rate. This result, together with the preceding data, indicated that the principal effect of phage Mu1 was not to select for spontaneous mutants but rather to induce new mutations in infected bacteria.

(*b*) *Extrinsic mutagenic agents:* The following tests were performed to determine whether the mutagenic activity of phage Mu1 lysates resided in the virus particles or in other substances, e.g., hypothetical base analogues produced as by-products of phage nucleic acid synthesis. In the latter instance it was expected (1) that purification of the crude lysates would reduce their mutagenic activity, and (2) that unpurified lysates would be mutagenic even under conditions precluding infection and subsequent lysogenization of bacteria. Phage Mu1 was partially purified by two cycles of centrifugation (1 hr at 20,000 × *g*) and resuspension in fresh L broth. This preparation induced mutations in sensitive bacteria at about the same frequency as unwashed lysates. When phage-resistant mutants unable to adsorb Mu1 and lysogenic bacteria able to adsorb but not propagate Mu1 were

TABLE 3

RATE OF MUTATION TO PHAGE T1 RESISTANCE*

Control			Phage Mu1		
Time (min)	Av no. of bacteria per plate ($\times 10^{-8}$)	Av no. of $T1^r$ colonies per plate	Time (min)	Av no. of bacteria per plate ($\times 10^{-6}$)	Av no. of $T1^r$ colonies per plate
0	0.26	17.5	60	2.2	6
70 (t_1)	0.65	14	90	2.3	18
120	2.5	33	120 (t_1)	5.4	21
180 (t_2)	23.2	74	180	20.8	43.5
240	120.0	168	240 (t_2)	83.3	149
Mutation rates†	1.8×10^{-8}			1.14×10^{-6}	

* Exponential phase cells of AB1172 were spread on PL plates either directly (control) or after a 20-min incubation at 37°C with phage Mu1 at a multiplicity of 5. Plates were incubated for the indicated times and then assayed for total cells and number of $T1^r$ clones; each datum is the average of two plate assays. Plates with Mu1-infected bacteria were first assayed 60 min after infection (30 min after plating).

† Mutation rates during exponential growth were computed from Newcombe's[16] equation: mutations/bacterium/division $= \ln 2(m_2 - m_1/b_2 - b_1)$, where m_1, m_2 are the number of $T1^r$ clones, and b_1, b_2 the total number of bacteria at times t_1 and t_2, respectively.

both exposed to droplets of unpurified lysate on PL plates as previously described, no induced mutants were found among several thousands of colonies subsequently assayed on EMM plates. These observations supported the view that phage Mu1 was in fact the active mutagen. They further showed that mutation induction depended not only on infection but on primary lysogenization of sensitive bacteria as well.

(*c*) *Genetic instability:* Abnormally high rates of spontaneous mutation have been recorded several times in certain strains of *E. coli* and *S. typhimurium*.[17–19] These unstable "mutator" strains often generate auxotrophic mutants at frequencies equal to, or greater than, those found in Mu1-infected bacteria. In at least two cases, instability is a hereditary character determined by discrete mutator genes.[19, 20] These observations suggested that phage Mu1 might induce mutations indirectly by creating an unstable condition similar to that of mutator strains. In effect, this would be an example of lysogenic conversion[21] to a mutator phenotype. This model became untenable, however, in view of the following dissimilarities between the two systems. First, mutator strains are persistently unstable so that multiple mutations accumulate rapidly, whereas phage Mu1 apparently induced high mutation rates only during the period immediately following infection and lysogenization. The spontaneous mutation rates to auxotrophy of established Mu1 lysogens were not demonstrably greater than those of wild-type *E. coli*. Second, mutants that arise in mutator stocks usually back-mutate rapidly, whereas the phage-induced mutants seldom reverted to prototrophy. Only two out of 58 auxotrophs screened so far produced measurable numbers of spontaneous revertants in populations of 10^9 to 10^{10} bacteria. Attempts to induce reversions in the stable auxotrophs with the mutagen, diethyl sulfate, also gave negative results. Third, the introduction of a mutator gene into an otherwise relatively stable auxotrophic strain can cause the back mutation rate to increase sharply,[22] whereas Mu1 stimulated forward mutation rates only. Seven nonlysogenic, UV-induced auxotrophic mutants were infected with Mu1, assayed for the number of surviving bacteria, and plated on EMM to select for colonies of prototrophic revertants. In all seven cultures, the number of colonies recovered per infected bacterium plated did not differ significantly from the corresponding ratios in parallel uninfected cultures.

(*d*) *Direct interaction between prophage and chromosomal genes:* The data of the preceding sections have shown that lysogenization is a requisite step for mutation induction. This suggested that mutations might result from a physical association between host genes and the prophage of Mu1. Direct evidence to support this hypothesis was obtained from experiments to determine the chromosomal site of the prophage. A nonlysogenic prototrophic Hfr strain of *E. coli* was crossed to six lysogenic F^- recipients that carried single phage-induced mutations at six different loci and several UV-induced auxotrophy markers as well. Analysis of recombinant progeny from these crosses showed that a high proportion (85–100%) of recombinants that inherited the Hfr wild-type alleles of the six phage-mutated genes were also nonlysogenic; conversely, 100 per cent of recombinants that inherited the F^- mutant alleles were lysogenic. This marked restriction on genetic recombination between the determinant of lysogeny (i.e., the prophage) and the six mutant loci showed that the prophage was closely linked to the site of phage-induced mutation in each of the F^- recipients. The linkage data were confirmed in reciprocal crosses

and in transduction tests which will both be described in detail in a later publication.

Discussion.—The evidence presented has shown that phage Mu1 induces a marked, though short-lived, increase in the forward mutation rates of infected nonlysogenic bacteria. The results of recombination analyses showing that Mu1 prophages are always closely linked to the sites of mutation support the working hypothesis that mutations occur when the phage is integrated at (or near to) the loci of functional host genes.

Phage Mu1's unique propensity for stable integration at many positions in the bacterial chromosome places it at one extremity in a spectrum of known episomic affinities for chromosomal sites. Transducing phage 363, which appears to have no fixed prophage site,[1] is at one end of the spectrum. In between are the prophages of lambda and 12 others which have only one specific site,[1] the prophage of P2 which has two or possibly three sites,[23] and the sex factor, F, of *E. coli* which has at least ten different sites.[1, 24]

Genetic modifications of episomes that restrict their capacity for integration to single chromosomal locations have been reported for both the F factor[25] and phage P2.[26] Mu1 phages released from auxotrophic lysogens have been tested several times for preferential integration at their former chromosomal sites, but no restricted variants have been found to date. It is not presently clear whether Mu1 will exhibit any site specificity, even within the limits of a single gene. Preliminary experiments have shown that two independently derived auxotrophs sharing a common enzyme deficiency will recombine in a cross to yield nonlysogenic prototrophic progeny. This suggests that the prophages of the two mutants did not occupy identical sites in the defective gene, but additional evidence will be required to eliminate other equally plausible interpretations.

The observation that Mu1 lysogens are immune to further phage-induced mutations probably means that double lysogenizations do not occur frequently. This is the phenomenon of prophage incompatibility which has been observed in several other systems.[27, 28] When homologous phages infect lysogenic cells, the viruses are not replicated and only rarely are integrated along with the established prophage. If one accepts the plausible assumption that superinfecting Mu1 phages are not readily integrated, the basis for this incompatibility cannot be competition for limited chromosomal sites, as is generally believed to be the case in other systems.

Efforts to gain further understanding of the exact cause of phage-induced mutation will necessarily involve studying the mechanism of phage integration. This mechanism remains unresolved for other phages at the present time; however, if the view that loss of cellular functions in Mu1 lysogens is a direct consequence of phage integration at functional loci is correct, this system will be an ideal one for testing some of the current models for integration.[1, 2, 4] Thus, it would be germane to know whether the genetic lesions in these mutants are chromosomal aberrations such as insertions or deletions, or whether the prophage represses functions without altering the structure of host genes. Information regarding the genetic fine structure of phage-mutated gene loci and information about the status of the prophage (present, absent, or altered) in prototrophic revertants will help to answer these questions. Experimental work in this direction is now actively in progress.

Phage Mu1's dual ability to occupy many chromosomal sites and to suppress the phenotypic expression of genes with which it becomes associated resembles the

"controlling elements" of maize more closely than any previously described bacterial episome. Although the system analyzed by McClintock[29] in maize is much more complex than the present system, it is interesting to note the similarities. First, controlling elements move from one position to another in the plant chromosomes. Second, when they are located close to a recognizable gene, they may, among other things, modify its phenotypic expression sometimes to the point of completely suppressing its normal function.

Hill[30] recently suggested that integration of a hypothetical episome at a tryptophan suppressor locus interfered with phenotypic expression of the gene. The present evidence that a bacterial episome is actually capable of preventing normal expression of host genes lends further credibility to this hypothesis.

Alikhanian and Iljina[31] reported presumptive mutagenic activity for an actinophage of *Actinomyces olivaceus*. Infected cultures produced morphological and nutritional variants at extremely high frequencies; however, the traits that appeared in lysogenic strains seemed to reflect principally the genotypes of cultures from which the phage was derived. The authors tentatively concluded that a process similar to phage-mediated transduction was involved. In the absence of further information, it is difficult to assess the degree of similarity, if any, between the actinophage-induced modifications and the Mu1 system.

Summary.—A new temperate bacteriophage (phage Mu1) of *E. coli* strain K12 is described. Sensitive bacteria infected with Mu1 exhibit increased forward mutation rates at many gene loci. The prophage of Mu1 is always closely linked to the chromosomal sites of phage-induced mutation. It is proposed that loss of genetic functions is a direct consequence of phage integration at functional loci. The potential uses of this system for studying the mechanism of integration are discussed.

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*EXCLUSION OF AN FLAC EPISOME BY AN HFR GENE**

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In a previous paper[1] we have described experiments which suggested that the presence of an Hfr gene excluded the acceptance of an Flac episome during conjugation. In these experiments a strain of *Escherichia coli* B which had been infected with Flac$^+$ was used as the chromosomal donor, and the Hfr strain AB 312, converted temporarily to the F$^-$ state (F$^-$ phenocopy), acted as the recipient. The offspring of such a cross were either Flac$^+$, F$^-$, or Hfr in mating type, but never Hfr-Flac$^+$.

A question that was not answered in the previous paper was whether or not the transfer of the chromosome during the mating prevented the simultaneous transfer of Flac$^+$; if this were the case, it would not be necessary to implicate a specific action of the Hfr gene present in the recipient. In this paper it will be demonstrated that simultaneous transfer of Flac$^+$ and chromosome can occur into an F$^-$ strain, as has already been suggested by the experiments of Adelberg and Burns,[2] and that therefore physical impedance by the chromosome is not responsible for the observed exclusion. This having been established, an experiment was carried out in which a mating between an Flac$^+$ donor and an Hfr recipient was interrupted after sufficient time to permit chromosomal transfer from the Flac$^+$ strain into an Hfr strain in F$^-$ phenocopy. Under these conditions, the Flac$^+$ factor, although able to be transferred, did not appear among the recombinants.

Another question that remained unanswered in the previous paper[1] was whether or not the Flac$^+$ episome ever entered the Hfr recipient. Flac$^+$ offspring were obtained, but no tests were carried out to ascertain that Flac$^+$ was actually transferred into the Hfr recipient. The Flac$^+$ offspring might, in fact, have resulted from the entrance of the Hfr chromosome into the Flac$^+$ strain. In the experi-

ment reported in this paper, reverse transfer from the Hfr into the Flac$^+$ parent was eliminated. Thus, all recombinants resulted from entrance of the chromosome of the Flac$^+$ parent into the Hfr parent. Under these conditions, transfer of the Flac$^+$ factor was found to be a rare event.

Materials and Methods.—The media, mating conditions, and tests used have been described.[1] The test for Flac$^+$ was simplified so that many strains could be tested with ease. Abbreviations used are: ade, adenine; arg, arginine; his, histidine; leu, leucine; met, methionine; pro, proline; thi, thiamine; thr, threonine; lac, lactose; sm^r, streptomycin-resistant; sm^s, streptomycin-sensitive; sf, sex factor.

Test for Flac$^+$: Presumptive Flac$^+$strains were crossed with lac$^-$ recipients which differed from them in being sm^r (see abbreviations above). Logarithmically growing cultures containing 10^9 cells per ml in broth were mixed in 25-mm diameter tubes in the proportion of 1.0 ml of the Flac$^+$ parent, 0.5 ml of the F$^-$ parent, and 1.5 ml of neopeptone broth. The suspension was incubated at 37° with gentle shaking. The conjugation was interrupted by rapid agitation of a 100-fold diluted aliquot at zero time and after 60 min. The treated samples were streaked directly on MacConkey lactose agar containing 100 γ of streptomycin per ml. Flac$^+$ strains gave rise to lac$^+$ colonies in the streaks of the 60-min samples, but not in the zero-time samples.

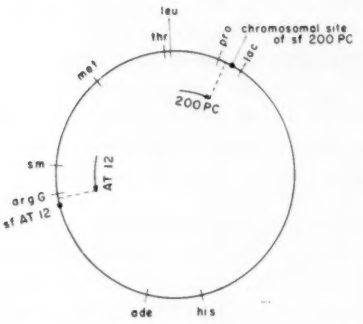

FIG. 1.—Linkage map of the pertinent genes.

Strains: Hfr strain AT 12 (see Fig. 1 for location of sex factor), which is met$^-$, ade$^-$, lac$^-$, T6^r, and sm^s, was obtained from Dr. A. J. Clark. Strain AB 1353 (thi$^-$, arg$^-$, pro$^-$, his$^-$, lac$^-$, T6^r, F$^-$) was obtained from Dr. A. Taylor. The Flac$^+$ donor, 200 PC (thi$^-$, thr$^-$, leu$^-$, T6^s, sm^r), was obtained from Dr. F. Jacob.

Results.—1. *Flac$^+$ transfer during chromosomal transfer:* This experiment was designed to test whether or not the chromosome and an Flac$^+$ episome can enter together from an Flac$^+$ donor into an F$^-$ recipient. Strain 200PC (thi$^-$, thr$^-$, leu$^-$, T6^s, Flac$^+$) was crossed with strain AB 1353 (thi$^-$, arg$^-$, his$^-$, pro$^-$, lac$^-$, T6^r, F$^-$). The mating was interrupted by killing 200PC, the minority parent, with phage T6 at times zero, 60 min, and 150 min. The following recombinant types were selected on minimal glucose agar: thr$^+$, leu$^+$, pro$^+$ at zero and 60 min, and thr$^+$, leu$^+$, his$^+$ at zero and 150 min. Colonies of the two types were picked and purified by two single colony isolations on the appropriate selective media. They were then tested for ability to ferment lactose on MacConkey agar, and for maleness by replica plating of a master plate onto two successive lawns of a suitably marked F$^-$ strain.[3] The results of this experiment are presented in Table 1. It can be seen that the majority of the chromosomal recombinants tested were lac$^+$ and males. Furthermore, most of the lac$^+$ strains segregated lac$^-$ strains with the same frequency as the Flac$^+$ parent. Thus, it can be concluded that most of the recombinants were Flac$^+$, and that therefore the chromosome

TABLE 1

FLAC+ TRANSFER DURING CHROMOSOMAL TRANSFER FROM FLAC+ STRAIN 200PC INTO F− STRAIN AB 1353

Type of selection	Time (min)	Frequency of recombinants (per cent male input)	Frequency of lac+ strains among chromosomal recombinants (per cent)	Frequency of males among lac+ strains (per cent)
thr+, leu+, pro+	60	4	68	86
thr+, leu+, his+	150	0.01	84	—

and the Flac+ episome can be transferred together.

About 4 per cent of the tested recombinants were of a peculiar lac type. They were lac− but gave rise to lac+ clones with relatively high frequency. This was a heritable characteristic, for reisolated lac− clones continued to give rise to lac+ clones with an approximate frequency of 1:100. Furthermore, they were "weak" males, i.e., weaker than males of the Flac+ type and rather resembling F+ males. No explanation is available for the origin of these strains.

2. *Flac transfer into Hfr strains:* Having determined that the chromosome and an episome can enter together into an F− recipient, a similar experiment was now carried out between an Flac+ donor and an Hfr recipient to see if the Hfr strain can at all receive the Flac+ episome. Strain 200PC (thi−, thr−, leu−, T6s, smr, lac−, Flac+) was crossed with strain AT 12 (ade−, met−, lac−, T6r, sms, Hfr) in F− aeration-phenocopy. The mating was interrupted by killing the donor, 200PC, at zero time and 150 min with phage T6. Two kinds of recombinants were selected for at these times: thr+, leu+, met+ and thr+, leu+, ade+. Of these, recombinants to be tested for other genetic characters were purified by two single colony isolations on the appropriate selective media. The results of this experiment are presented in Table 2. It can be seen that in contrast with the results obtained with an F− recipient, most of the recombinants were lac−. Therefore, there is a definite exclusion of the Flac+ episome by the Hfr gene in the recipient.

All the recombinants that had been purified were tested for the other, unselected auxotrophic character, i.e., all thr+, leu+, met+ recombinants were tested for adenine requirement, and all thr+, leu+, ade+ recombinants were tested for methionine requirement. When met+ was selected for from the donor, most of the colonies (47/48) were ade− as expected, since ade+ enters after met+ from 200PC. When ade+ was selected for from the donor, only 17 out of 64 colonies had also received met+, indicating a preferential inheritance of the Hfr chromosome of the recipient. In fact, out of 112 recombinants tested only three did not inherit the AT 12 sex factor A possible explanation for this unexpected preferential inheritance is that the sex factor of the recipient is a starting point for DNA replication, even in the state of F− phenocopy.

Of the lac+ recombinants, all but 2 out of 13 did not inject Flac+ and did not

TABLE 2

RECOMBINANT TYPES IN A CROSS BETWEEN FLAC+ DONOR 200PC AND HFR RECIPIENT AT 12

Type of selection	Frequency of recombinants (per cent male input)	Frequency of lac+ strains among recombinants (per cent)	Frequency of males among recombinants (per cent)
thr+, leu+, met+	0.15	8	97
thr+, leu+, ade+	0.02	14	97

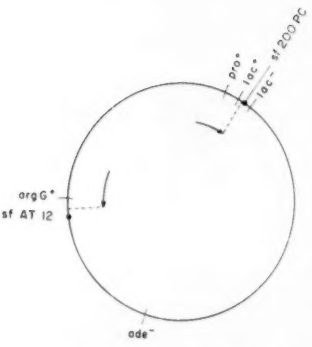

FIG. 2.—Proposed genetic structure of strain a30.

inject pro+ early. These 11 were Hfr offspring of the AT 12 type and not Flac+. The other two exceptional recombinants, a30 (ade−, sm^s) and m16 (met−, sm^s), segregated lac− clones, a30 with much higher frequency than m16. Therefore, they both appeared to be diploid for the lac region. Strain m16 injected lac+ with a frequency of about 50 per cent after 60 min, and pro+ early like the Flac+ parent. It did not inject arg G early. It can thus be concluded that m16 is an Flac+ strain, in which the sf AT 12 was crossed out.

Strain a30 injected lac+ and pro+ early and with about equal frequencies (1%), as if they were both chromosomal markers, and arg G early, like strain AT 12. Therefore, this strain appeared to be a double male harboring two chromosomal sex factors, one of them between two lac regions (Fig. 2). To test this hypothesis, strain a30 (ade−) was crossed with AB 1353 (pro−, thi−, his−, arg−, lac−, F−) and lac+, ade+ recombinants were selected after 60 min of mating. The mating was interrupted by agitation. The unselected marker pro+ was scored among the lac+ recombinants, and was found to occur among them with a frequency of 66 per cent. This supports the hypothesis that strain a30 injects the sequence lac-pro . . . . . sf AT 12, as well as the sequence arg G- - - - - pro-lac-sf 200PC. Furthermore, the lac+ recombinants were scored for maleness and only 11 per cent were males. All of the latter were of the Flac+ type. Therefore, it was confirmed that most of the time strain a30 injects lac+ as an early chromosomal marker and only in 11 per cent of the lac+ recombinants is the lactose region injected as an episome.

Discussion.—The experiments presented in this paper show that an Hfr recipient excludes Flac+ from an Flac+ donor. However, the lac+ allele can be recovered on the chromosome in about 10 per cent of the recombinants. Evidence for the mechanism of this chromosomal lac+ recovery has been obtained by Scaife and Gross.[4] They have shown that when an Flac+ episome activates injection of a chromosome carrying lac−, a recombination occurs between the episome and the chromosome in such a way that the chromosome is donated with the sequence lac+-pro. . . . . lac− sf 200PC (cf. Fig. 2). Thus, it is to be expected that in any cross involving an Flac+ donor, lac+ can be recovered as an unselected marker in a certain fraction of recombinants as a chromosomal gene.

Furthermore, the mechanism proposed by Scaife and Gross explains how the double male a30 was obtained in the cross between the Flac+ donor 200PC and the Hfr recipient AT 12. The lac+ allele entered early and on the chromosome, while the lac− allele and sex factor also entered with the donor chromosome at the very end of the injection. Such a strain, with the lac region in duplicate, is somewhat unstable and often loses one of the lac alleles during replication. This would explain why the strain segregates lac− clones. Strain a30 presumably gives rise also to Flac+ episomes since occasionally it donates the Flac+ episome to an F−

recipient during mating. However, it appears that in strain a30 these episomes are either not replicated or destroyed since $Flac^+$ is donated with low frequency. It is reasonable to attribute this loss of $Flac^+$ to the presence in strain a30 of the AT 12 sex factor. In fact, this phenomenon would parallel the observed exclusion of $Flac^+$ by the sex factor of Hfr recipients.

The author would also like to propose that the only true $Flac^+$ recombinant (m16) obtained arose exactly like a30. Lac^+ came in early on the donor chromosome, and lac^- followed by the sex factor came in at the end. However, since the Hfr gene of the recipient was crossed out, this strain can harbor a replicating episome. We have preliminary evidence which suggests that strain m16 does have a duplicate lac region on its chromosome.

We thus arrive at the conclusion that an Hfr recipient completely excludes an incoming $Flac^+$ episome. A similar exclusion of $Flac^+$ by a strain carrying $Fgal^+$ has been observed by Echols.[5] He suggests that the excluded episome either does not enter or is destroyed after entrance. The present results favor the hypothesis of destruction. This is suggested by the behavior of strain a30 in which, because of the presence of Hfr, $Flac^+$ is expressed only rarely. In this strain absence of $Flac^+$ cannot be a case of nonentry. The phenomenon described here may be analogous to the restriction of phage λ by phage P_1, studied by Dussoix and Arber.[6] In this case Dussoix and Arber have shown that the incoming DNA is destroyed. It is interesting to speculate that destruction of "foreign" DNA is a general mechanism that plays a role in maintaining the integrity of the cell's genetic apparatus.

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† U.S. Public Health Service trainee in genetics.

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*PHOTOREVERSIBILITY OF INDUCED MUTATIONS IN A NONPHOTOREACTIVABLE STRAIN OF ESCHERICHIA COLI**

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The photoreversal of mutations to prototrophy induced by ultraviolet light (UV) and the photoreversal of UV killing are differentially affected by acriflavine.[1] One possible explanation is that the lesions leading to photoreversible prototrophy differ from those leading to photoreversible death, and that they may be photoreversed by distinct mechanisms. The isolation of a mutant strain of *Escherichia coli* B that is unable to photoreverse UV killing[2] makes possible a direct test of this hypothesis. The "photoreactivating enzyme" normally produced by *E. coli*[3] is not obtainable from the mutant strain,[4] which suggests that this

enzyme is largely responsible for the photoreversal of prelethal UV damage. Should the nonphotoreactivable strain be found to retain the ability to photoreverse induced prototrophy, it would be apparent that the "photoreactivating enzyme" is not required for the photoreversal of these mutations. This report describes experiments to determine the ability of the nonphotoreactivable strain to photoreverse UV-induced mutations to prototrophy and, by way of comparison, to streptomycin resistance.

Materials and Methods.—A radiation-resistant variant of Harm's nonphotoreactivable strain, designated here as H/*r*, was obtained from Dr. Ruth F. Hill. This strain was used in experiments involving mutations to streptomycin resistance (*str-r*). An argenine-requiring substrain of H/*r* (H/*r*30), isolated after UV irradiation and penicillin screening, was used in studies of induced prototrophy (*arg*$^+$). Methods of growing cultures, irradiating with UV, and exposing to photoreversing light (PRL) were as described elsewhere.[1] All exposures to PRL were for 10 min. Mutations to prototrophy were detected by plating on SEM agar (minimal agar supplemented with 5 per cent liquid nutrient broth), and the same medium was used to assay the survival of the argenine-requiring parent strain. Assays for mutations to streptomycin resistance were made by a method previously described in detail.[5] The resistant mutants were selected in nutrient agar containing 100 units/ml of streptomycin (allowing 3 hr of incubation before adding the antibiotic, to ensure full phenotypic expression of mutations to resistance). Assays for survival of the streptomycin-sensitive parent strain were made on nutrient agar without streptomycin (Difco nutrient agar with 0.5% NaCl added). Incubation was at 37 °C for 2 days (*arg*$^+$) or 5 days (*str-r*). Streptomycin was generously supplied by Chas. Pfizer and Co.

Results.—Figure 1 shows survival and frequency of induced mutations to streptomycin resistance in strain H/*r*, and in its photoreactivable counterpart strain B/*r*, with and without PRL posttreatment. Normal photoreversal of both killing and induced mutation to streptomycin resistance was obtained in strain B/*r*. In strain H/*r*, however, photoreversal was obtained neither for killing nor for induced streptomycin resistance. The loss of the ability to photoreverse these mutations clearly accompanies the loss of the ability to photoreverse killing.

Figure 2 shows survival and frequency of induced prototrophs in strain H/*r*30, in the dark and after PRL posttreatment, with survival of strain B/*r* under the same conditions shown for comparison. In these experiments, a small but statistically significant elevation of survival after PRL posttreatment was observed in the "nonphotoreactivable" strain. This slight residual photoreversal of killing was found only when plating was on SEM agar, never on nutrient agar. It was observed, under these conditions, in strain H/*r* and in Harm's original strain, as well as in the auxotrophic strain H/*r*30. It was not obtained, even on SEM agar, when the source of PRL emitted primarily long ultraviolet light of 3550 A (a peak in the action spectrum for photoreactivation[6]) instead of white light. Whatever the explanation of this effect, it is clear that most of the photoreactivation normally obtained in strains of the B/*r* type does not occur in this strain. In contrast, photoreversal of induced *arg*$^+$ mutations is a large effect, similar in its order of magnitude to the photoreversal of induced prototrophy observed in derivatives of strain B/*r*.[1] This photoreversal is not diminished by incubating the population

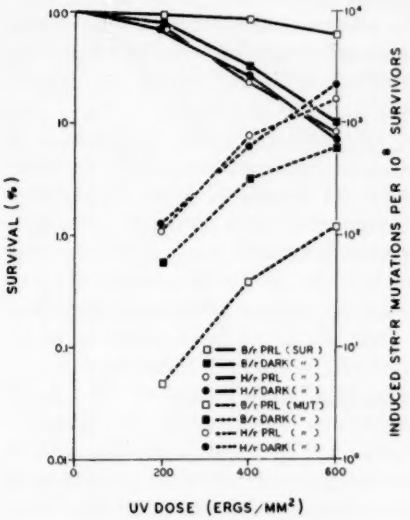
SURVIVAL (%)
INDUCED STR-R MUTATIONS PER 10⁸ SURVIVORS
UV DOSE (ERGS/MM²)
B/r PRL (SUR)
B/r DARK (")
H/r PRL (")
H/r DARK (")
B/r PRL (MUT)
B/r DARK (")
H/r PRL (")
H/r DARK (")

FIG. 1.—Photoreversal of UV killing and UV-induced mutations to streptomycin resistance in strains B/*r* and H/*r*. Titers at 100% survival: 1.2×10^8 bacteria per ml (B/*r*) and 1.3×10^8 bacteria per ml (H/*r*). Each point is the average of three similar experiments.

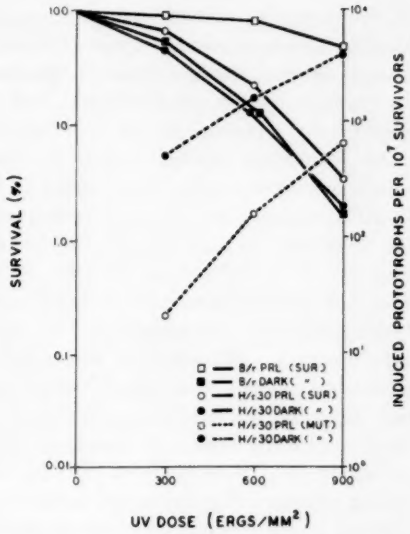

FIG. 2.—Photoreversal of UV killing in strains B/*r* and H/*r*30, and of UV-induced mutations to prototrophy in strain H/*r*30. Titers at 100% survival: 1.3×10^8 bacteria per ml (B/*r*) and 1.4×10^8 bacteria per ml (H/*r*30). Each point is the average of three similar experiments.

on nutrient agar during the first 90 min after treatment with UV and PRL (the critical period during which the mutation frequency is "fixed"[7]) nor by the use of near ultraviolet light instead of white light for PRL posttreatment. Thus, while photoreversal of killing is virtually absent in this strain, mutations to prototrophy remain normally photoreversible.

Discussion.—The loss, by mutation, of the "photoreactivating enzyme" results in loss of the ability to photoreverse UV-induced mutations to streptomycin resistance, as well as loss of the ability to photoreverse most UV killing, but does not prevent the photoreversal of UV-induced prototrophy. These results indicate the existence of at least two kinds of photoreversal, and, by implication, at least two kinds of UV-initiated mutagenesis.

The unique nature of the UV-produced lesions leading to induced prototrophy has already been suggested by their unusual responses to various pre- and post-irradiation influences. In particular, UV-induced prototrophy is subject to "dark repair" (the irreversible loss of potential induced prototrophs, in the dark, when postirradiation protein synthesis is inhibited).[7] Neither UV killing (in strains of the B/*r* type) nor UV-induced streptomycin resistance is reversed by postirradiation incubation with chloramphenicol,[5] or by other conditions promoting the irreversible loss of over 95 per cent of the UV-induced prototrophs by "dark repair." Furthermore, UV-induced mutations to prototrophy are subject to photoprotection[1] (the reduction of UV effects by preirradiation exposure to visible light). Neither UV killing (in strains of the B/*r* type) nor UV-induced mutation

to streptomycin resistance shows a comparable response to photoprotecting light. Mutant strains showing reduced efficiency in all three kinds of repair suggest that a single enzyme, distinct from the "photoreactivating enzyme," may be responsible for photoprotection, photoreversal, and "dark repair" in the case of UV-induced prototrophy, a possibility now under investigation in this laboratory.

An interesting aspect of these results is the implication that mutation must sometimes involve more than changes in the linear sequence of nucleotides in DNA. If all mutations were simply coding changes (of greater or lesser magnitude), the occurrence of a specific kind of lesion characteristic of a particular *phenotypic class* would be difficult to explain. It has been suggested that auxotrophy may arise not only by structural gene mutations but also by the attachment of "controlling elements" or episomes[8, 9] at the locus in question, with resulting loss of gene function. Mutations to prototrophy, then, would involve detachment of the repressing element, and "repair" would involve reattachment. It has been pointed out[9] that the postirradiation conditions promoting "dark repair" of UV-induced prototrophy are strikingly paralleled by conditions that interfere with prophage induction by UV in lysogenic strains of bacteria. To these similarities may be added the fact that both UV-induced prototrophy and prophage induction are subject to photoprotection.[1, 10] A major difference, however, is the frequency of occurrence, which approaches 100 per cent at low doses of UV for prophage induction, and rarely exceeds 10^{-3} for induced prototrophy, a frequency typical of true mutagenic events. This, and the ready inducibility of prototrophy by base analogues,[11] suggests that coding changes are involved. Classes of genetic changes in which true mutagenic events and the action of "controlling elements" may occur as coordinated phenomena have been described.[12]

Another unusual feature of UV-induced prototrophy susceptible of "dark repair" is its association with "supersuppression."[13] Such prototrophy is often due to the action of a suppressor mutation capable of suppressing a wide and nonspecific array of independent auxotrophic markers.[14] It seems possible that the mechanism of "supersuppression" is related to the apparently unique responses of UV-induced prototrophy to conditions promoting photoprotection, photoreversal, and "dark repair."

Summary.—A radiation-resistant derivative (strain H/*r*) of the nonphotoreactivable mutant strain isolated by Harm was found to be incapable of photoreversing UV-induced mutations to streptomycin resistance, as well as UV killing. Photoreversibility of UV-induced mutations to prototrophy, however, was found to be essentially normal in an argenine-requiring substrain of H/*r* (H/*r*30). It was concluded that photoreversal of UV-induced mutations to prototrophy does not require the "photoreactivating enzyme."

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*THE ROLE OF RNA IN REPRESSION OF ENZYME SYNTHESIS**

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It is known that chloramphenicol (CAP) inhibits protein synthesis by interfering with the transfer of amino acids from sRNA to ribosomes.[1] In agreement with the general effect of CAP on protein biosynthesis, the synthesis of numerous individual enzymes is inhibited by this antibiotic.[2] Working with low concentrations of CAP, it was demonstrated that the synthesis of various repressor-controlled enzymes was affected to a greater degree than the formation of total protein or constitutive enzymes.[3, 4] The preferential inhibition by CAP of the synthesis of β-galactosidase, a repressor-controlled enzyme, is attributed to the reduced rate of formation of β-galactosidase messenger RNA.[4] Therefore, CAP preferentially inhibits enzyme synthesis by the same mechanism involved in enzyme repression.

Not only is the CAP-promoted repression a general effect for repressor-controlled enzymes, but it requires a functional regulator gene for the appropriate enzyme. Thus, CAP does not repress β-galactosidase or tryptophan synthetase in strains mutant for the respective regulator genes.[5] Furthermore, the CAP-promoted repression of inducible enzymes is not a manifestation of catabolite repression, nor does the repression of biosynthetic enzymes result from increased pools of biosynthetic end products.[5]

The participation of a functional regulatory system in the CAP-promoted repression of enzyme synthesis indicates that CAP elicits the increased production of specific repressor molecules within the cell. With the goal of obtaining evidence on the nature of the molecules which might be responsible for CAP-promoted repression, some of the metabolic changes which occur during CAP treatment have been examined. The data of this report demonstrate a consistent correlation between high relative rates of RNA synthesis, concomitant with the repression by CAP of β-galactosidase synthesis. These findings support the hypothesis[6] that certain polyribonucleotides are involved as repressors of enzyme synthesis.

Materials and Methods.—Bacterial cultures and media: The following strains of *Escherichia coli* were employed: ML30, inducible for β-galactosidase; $15T^-U^-M^-$ (from T. D. Brock), inducible for β-galactosidase and auxotrophic for thymine, uracil, and methionine; K-12: 3000, inducible for β-galactosidase; and K-12: 3300 (from F. Jacob), constitutive for β-galactosidase. Experiments were usually conducted in a mineral salts medium with 0.5% glycerol as carbon source.[4]

Shift-up experiments were conducted by first growing the bacteria in minimal medium supplemented with arginine, leucine, glycine, alanine, valine, threonine, isoleucine, and lysine. To effect the shift, vitamin-free casamino acids (0.1%), L-tryptophan (50 μg/ml), and the ribonucleosides uridine, cytidine, guanosine, and adenosine (each at 25 μg/ml) were added to the medium. The final differential rates of β-galactosidase synthesis were the same in both media.

Shift-down experiments were conducted by first growing the bacteria in glycerol-salts medium supplemented with 200 μg/ml L-serine, in which NH_4^+ and serine provided the nitrogen sources. The bacteria were removed from the medium by filtration, and then resuspended in nitrogen-free salts medium with L-serine as the sole nitrogen source. This procedure resulted in an uninterrupted shift in the growth rate from 0.60 doublings/hour to 0.45 doublings/hour for bacteria growing in the absence of CAP.

Biochemical and enzymological methods: β-galactosidase activity was determined as previously described.[4] Protein was determined by the method of Lowry *et al.*,[7] and RNA was assayed by the orcinol method,[8] using purified yeast RNA as a standard.

Inhibitor studies: Experiments with bacteria growing logarithmically were performed as described by Sypherd and DeMoss.[5]

For resting cell studies, the bacteria were depleted of thymine, uracil, or methionine by a 45-min incubation, with vigorous shaking in the absence of the particular nutrient, followed by a similar 45-min incubation in the absence of both the nutrient and glycerol. Following the successive starvation periods, growth could be initiated only by providing the suspension with both the nutrients for which they were starved. Protein synthesis in resting cells was determined from the quantity of C^{14}-leucine incorporated into TCA-insoluble material. β-Galactosidase was induced with methyl-β-D-thiogalactoside (TMG), at a final concentration of 10^{-3} *M*. In some cases, isopropyl-β-D-thiogalactoside (IPTG) was used, but there was no qualitative difference in the results with either TMG or IPTG. CAP was used in a concentration which would reduce the rate of C^{14}-leucine incorporation to 50–60% the control rate (*ca.* 0.8–1.2 μg/ml).

Results.—The addition of CAP (0.8 μg/ml) to an exponentially growing culture results in an immediate shift to a lower growth rate. Accompanying this shift is a more severe reduction in the rate of β-galactosidase synthesis (Fig. 1*A* and *B*). Figure 1*B* also shows that the rate of DNA synthesis continues unabated, while RNA synthesis is accelerated by CAP. This latter phenomenon has been reported by others.[9, 10]

In view of the hypothesis that repressor levels are elevated in CAP-treated cells,[5] and the fact that the RNA synthesis is similarly stimulated, several experimental approaches were used to test the possibility that accelerated rates of RNA synthesis

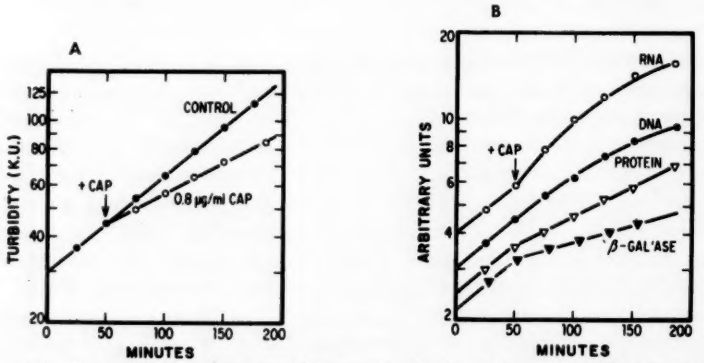

FIG. 1.—(*A*) The reduction of exponential growth rate by CAP. The antibiotic (0.8 μg/ml) was added to a culture of ML30 growing exponentially in glycerol-salts medium. (*B*) The synthesis of macromolecules in CAP-treated culture shown in (*A*).

were essential for the manifestation of CAP-promoted repression of β-galactosidase formation. The rates of RNA and β-galactosidase synthesis were determined during prolonged growth in CAP. It was shown previously that the CAP-promoted repression of β-galactosidase formation is released after approximately three generations in CAP.[4] This recovery of enzyme synthesis occurs even though the rates of growth and total protein synthesis remain inhibited. Figure 2*A* and *B* shows a close correlation between the repression by CAP of β-galactosidase and the concomitant stimulation of RNA synthesis. It can also be seen that the rate of RNA synthesis declines to the normal, pretreatment rate just prior to the recovery of β-galactosidase formation.

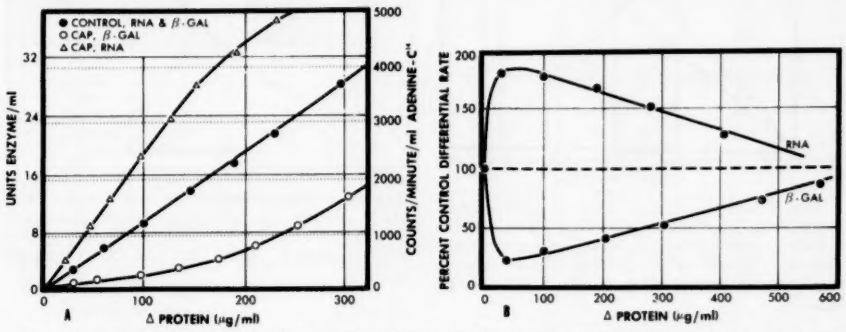

FIG. 2.—(*A*) The synthesis of β-galactosidase and RNA in CAP-treated and nontreated cultures of ML30 growing exponentially in glycerol-salts medium with 10^{-3} *M* TMG. Growth proceeded for over three generations after the addition of CAP. (*B*) The values for β-galactosidase and RNA in the CAP-treated culture in (*A*) are plotted as per cents of the respective rates in the nontreated culture.

It was previously shown[4, 5] that CAP does not repress β-galactosidase synthesis in nitrogen-depleted cells, which may indicate that a nitrogenous compound mediates the CAP-promoted repression. The polynucleotide nature of such a compound may be inferred from experiments with a thymine-less, uracil-less auxotroph. A strain of *E. coli* ($15T^-U^-M^-$), which requires thymine, uracil, and methionine for growth, was starved for each of the pyrimidines separately. The depleted cultures were then induced to synthesize β-galactosidase in the presence and absence of CAP. Figures 3 and 4 show that CAP elicited the repression of β-galactosidase synthesis only in the culture which was starved for thymine. Repression did not occur in the uracil-starved culture, even though leucine-C^{14} incorporation was inhibited 45 per cent. It was also found that when a culture of this organism was starved for methionine, CAP did not repress β-galactosidase synthesis. While this result must be interpreted cautiously, it is significant that RNA synthesis ceases when this organism is depleted of its methionine. The conclusion from these experiments is that uracil (and perhaps methionine) deprivation resulted in a loss of the CAP-promoted repression due to the limited RNA synthesis (or turnover) which occurs under these conditions. The experiments also indicate that a uracil-containing class of molecules mediates the CAP-promoted repression of enzyme synthesis.

The results obtained with the starved cultures support those obtained with growing cells: there is a correlation between the CAP-promoted repression of β-

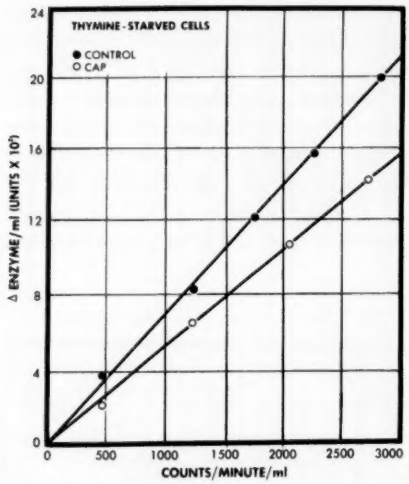

FIG. 3.—The relative rates of synthesis of β-galactosidase after induction with TMG (10^{-3} *M*) in a thymine-glycerol-starved culture of 15T⁻U⁻M⁻. C^{14}-Leucine incorporation was inhibited 40% in the CAP-treated culture.

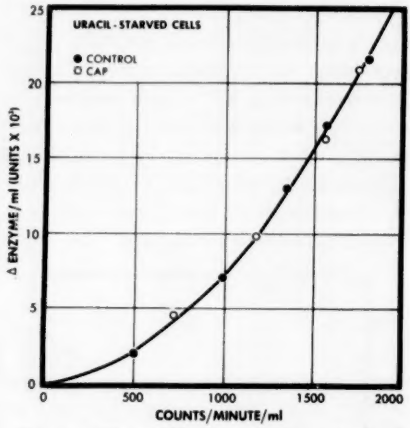

FIG. 4.—The relative rates of synthesis of β-galactosidase after induction with TMG (10^{-3} *M*) in a uracil-glycerol-starved culture of 15T⁻U⁻M⁻. C^{14}-Leucine incorporation was inhibited 45% in the CAP-treated culture.

galactosidase synthesis and the stimulation of RNA synthesis which occurs during CAP treatment. All these data suggest that increased rates of RNA synthesis are necessary for the CAP-promoted repression to occur. The obligatory nature of this relationship is suggested by experiments in which RNA synthesis in CAP-treated bacteria is halted. It is known that shifting a culture to a slower growth rate will result in the immediate cessation of RNA synthesis.[11] In such "shift-down" cultures, RNA formation resumes only after the synthesis of sufficient protein to establish the protein/RNA ratio characteristic for the new growth rate. Shift-down conditions (see *Materials and Methods*) similarly result in the cessation of RNA synthesis in CAP-treated cells (Fig. 5*A*). Figure 5*B* shows that following the shift and the subsequent cessation of RNA synthesis, the repression by CAP of β-galactosidase synthesis is released. Under these conditions, when RNA is no longer being made at an accelerated rate, the rate of β-galactosidase formation in the CAP-treated culture returns to the normal, pretreatment rate.

Thus, cessation of the accelerated rate of RNA synthesis in CAP-treated cells results in the release of the CAP-promoted repression of β-galactosidase synthesis. These data are in agreement with previous indications that RNA synthesis must proceed at an accelerated rate to maintain the repression by CAP of β-galactosidase synthesis.

The conclusion derived from the above experiments is that the CAP-promoted repression of β-galactosidase synthesis is the result of RNA synthesis proceeding at a disproportionately greater rate than total protein synthesis. Thus, it might be predicted that any condition which leads to accelerated rates of RNA synthesis will also elicit the repression of β-galactosidase synthesis. It is possible to establish conditions which yield accelerated rates of RNA synthesis in the absence of CAP.

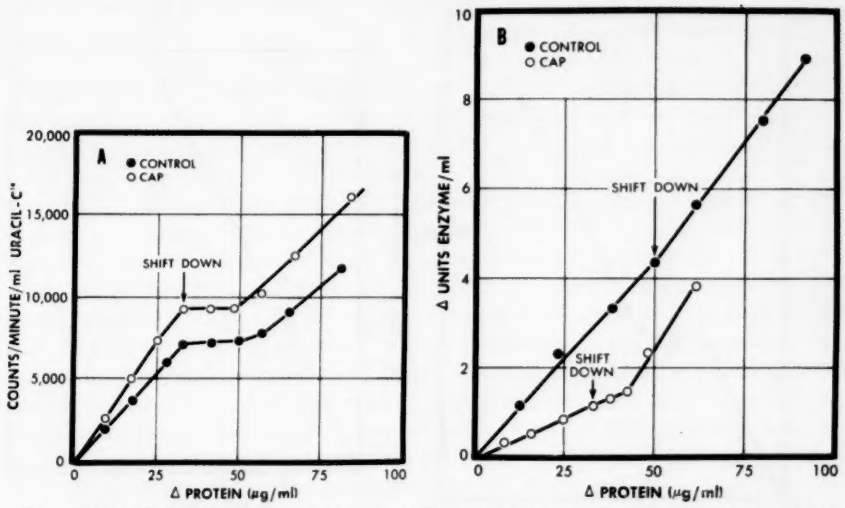

FIG. 5.—(*A*) Relative rates of RNA synthesis (uracil-C^{14} incorporation into TCA-insoluble material), before and after shifts to slower growth rates, of a CAP-treated and nontreated culture of ML30. (*B*) Relative rates of synthesis of β-galactosidase following shifts to slower growth rates of the cultures shown in (*A*).

For example, when the growth rate of a culture is abruptly increased (shifted-up), RNA synthesis will proceed at a greater rate than protein synthesis, until the RNA/protein ratio reaches that which is characteristic of the new growth rate.[11] If an accelerated rate of RNA formation is sufficient to elicit repression of β-galactosidase synthesis, it is predicted that in a culture which undergoes "shift-up," β-galactosidase synthesis will be repressed. This prediction was tested by subjecting a culture of K-12: 3000 (i+) to shift-up conditions (see *Materials and Methods*) in the presence of the inducer TMG. Table 1 shows that following the shift-up, RNA synthesis proceeds at a greater rate than total protein synthesis. In Figure 6, it can be seen that concomitant with the acceleration of RNA synthesis, there is a transient repression of β-galactosidase synthesis. This repression occurs during the time that the RNA/protein ratio is undergoing its greatest change (Table 1).

Before the conclusion could be made that shifting-up produces the same effect on β-galactosidase synthesis as does CAP, a similar experiment was performed with the β-galactosidase-constitutive mutant, K-12: 3300. β-Galactosidase syn-

TABLE 1

GROWTH RATES AND RNA/PROTEIN RATIOS BEFORE AND AFTER A SHIFT-UP IN GROWTH

Time after shift-up	K-12: 3000 (i+)		K-12: 3300 (i−)	
	k	RNA/prot.	*k*	RNA/prot.
−60	0.47	0.275	0.48	0.260
−30	0.47	0.273	0.48	0.260
0	0.47	0.275	0.48	0.260
15	1.0	0.355	1.1	0.342
30	1.1	0.485	1.2	0.477
60	1.1	0.580	1.2	0.560
80	1.1	0.575	1.2	0.568

k = growth rate in doublings/hour.

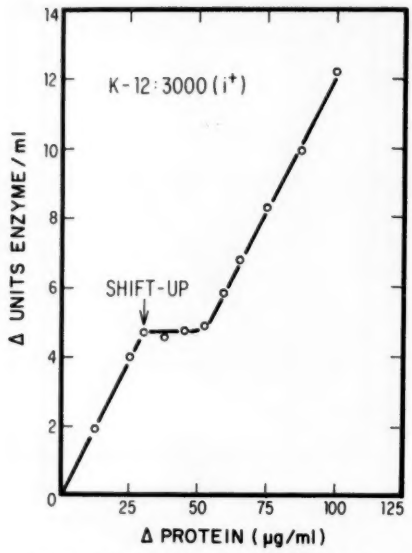

FIG. 6.—The effect on β-galactosidase synthesis of a shift to a higher growth rate (see *Materials and Methods*) of K-12: 3000 (i+).

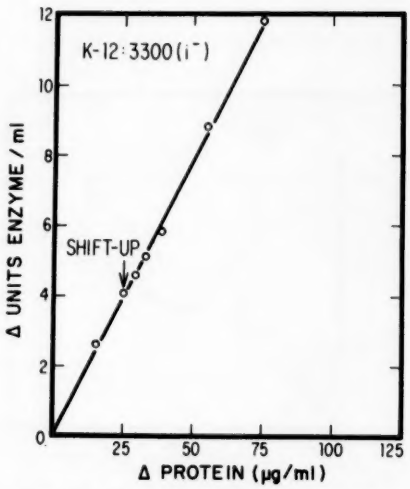

FIG. 7.—The absence of an effect on β-galactosidase of a shift to a higher growth rate of K-12: 3300 (i−).

thesis in this organism is resistant to repression by CAP.[5] If the repression elicited by shifting is similar to that elicited by CAP, shifting-up should not result in the repression of enzyme synthesis in this organism. The experiment was performed in the same manner as with the inducible organism, and the results are shown in Figure 7. Although the shift resulted in the same per cent increase in the growth rate and in RNA synthesis (Table 1), β-galactosidase synthesis in this mutant was not affected by the shift. It is evident, therefore, that the gene change i+ → i− in K-12: 3300 renders β-galactosidase nonrepressible both by CAP[5] and by a shift-up in growth rate. Since β-galactosidase synthesis in the i− mutant is susceptible to catabolite repression,[5] it may be concluded that the effects of a shift-up in growth on enzyme synthesis in the i+ strain (Fig. 6) are not due to catabolite repression. An interpretation of these experiments is that a shift-up in growth, like CAP treatment, produces accelerated rates of RNA synthesis and of the formation of specific repressor molecules. The resulting increase in repressor levels may lead to the repression of induced enzyme synthesis. However, in the constitutive mutant, which presumably makes no repressor, the acceleration of RNA synthesis, either by CAP treatment or shift-up in growth, is without effect on enzyme synthesis. In this way, the imbalance of macromolecule synthesis during shift-up may produce effects on β-galactosidase synthesis similar to those elicited by CAP.

Discussion.—The stimulation by CAP of total RNA synthesis has been shown by other workers, and provided much of the rationale for investigating its relationship to the CAP-promoted repression of β-galactosidase. The results from the present study show a clear inverse relationship between the rates of synthesis of RNA and of β-galactosidase in CAP-treated cells. In a previous study we showed that

the repression of β-galactosidase in exponentially growing cells may be elicited by certain antibiotics other than CAP,[4] but not by general growth inhibitors.[4, 5] It has also been found that total RNA synthesis is stimulated by all those antibiotics which elicit the repression of β-galactosidase.[12] We have been led to conclude that the repressive action of CAP is the result of an imbalance in the synthesis of macromolecules following antibiotic treatment, rather than a direct involvement of CAP in the regulatory system of β-galactosidase.

The fact that the CAP-promoted repression of β-galactosidase synthesis acts through the normal regulatory system[5] makes it attractive to postulate that CAP treatment results in a relative enrichment of specific repressor molecules. Furthermore, the finding that the repression of β-galactosidase synthesis is accompanied by the elevation of the internal concentration of RNA leads us to propose that a polyribonucleotide functions as a repressor component. It may be argued that among the polyribonucleotides accumulated during CAP treatment, there exists messenger RNA for a protein repressor. This could then lead to an increased rate of synthesis of a protein-repressor component. However, this possibility makes it necessary to postulate that the synthesis of such a repressor protein is less sensitive to inhibition by CAP than the synthesis of all other proteins. Therefore, the simplest interpretation of the data presented here is that the RNA acts directly.

The conclusion regarding the participation of RNA as a repressor component is necessarily tempered by the fact that a causal relationship between the stimulation by CAP of RNA synthesis and the CAP-promoted repression of β-galactosidase synthesis could not be unequivocally demonstrated. The possibility that specific repressors of enzyme synthesis are polyribonucleotide has been proposed by a number of workers in recent years.[6] However, Jacob *et al.*[13] have presented evidence, involving suppressor mutations, that the repressor of λ phage is polypeptide in nature. Similar evidence has recently been reported by Garen and Garen,[14] suggesting that the products of the regulator genes for alkaline phosphatase are protein. Monod *et al.*[15] have proposed that "allosteric" enzymes function as repressors of enzyme synthesis. Therefore, we suggest that repressor systems involve at least two components. One component, a protein, has specificities for a small molecule "effector"[6] and for a polyribonucleotide. The second component is the polyribonucleotide, with specificity for the operator gene or gene product.[16] One can readily develop models which place repressor function in a system involving both an RNA and a protein component. Thus, it is probable that the exact nature of all the components of specific repressor systems can be elucidated only in *in vitro* enzyme synthesizing systems.

Summary.—Several experimental approaches have shown that a close correlation exists between the CAP-promoted repression of β-galactosidase synthesis and the stimulation of RNA synthesis. These findings have led to the conclusion that a polyribonucleotide functions as a component of the regulatory system for β-galactosidase.

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† The data of the report were taken from a thesis submitted by the senior author to the Graduate

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*PERIODIC BEHAVIOR IN CHARGED MEMBRANES AND ITS PHYSICAL AND BIOLOGICAL IMPLICATIONS**

BY R. H. ARANOW

RIAS, BALTIMORE, MARYLAND

Communicated by S. Lefschetz, October 25, 1963

Periodic variations in solvent flow, electrical potential, concentration, and pressure in membranes can be explained as a consequence of the solution to the hydrodynamic equations of motion if all forces and boundary conditions are taken into account.

In the effort to explain the periodic behavior observed in the nerve model system of Teorell[1] where a silica gel membrane separated two stirred compartments containing electrolyte of the same species but of different conductance, Teorell used the equations of electrokinetics but ignored the gradient of chemical potential and the dependence of current flow on pressure. He introduced nonlinearity by deriving a relationship in which the steady-state resistance is a nonlinear function of bulk flow. He then introduced the *ad hoc* assumption that the rate of change of the instantaneous resistance is proportional to the difference of the instantaneous resistance from the steady-state value. With his equations he succeeded in describing quantitatively the results of his experiments.

We have succeeded in deriving all of Teorell's equations from first principles and in the process have shown that the periodic behavior is a consequence of the effect of boundary conditions on hydrodynamic stability.

The implications of the theory to be presented and of Teorell's experiment are

that similar periodic phenomena in biological systems may have their origin in hydrodynamic processes. It is exciting to speculate that such processes as the pulsation of the heart cell and the mechanism by which a stimulus triggers a nerve cell may be in the class of periodic hydrodynamic phenomena. The coupling of the hydrodynamic membrane process to chemical potential and thus to chemical reactions may in some cases account for the periodicity of chemical reactions. Thus, it is suggested that hydrodynamic stability studies may provide a fresh new outlook on the problem of the origin of periodic biological phenomena.

The important new feature which we have introduced into Teorell's problem is the fact that the clamping force on the membrane acts like a body force in the equations of motion.[2] The equations used[3] assume an isothermal quasi-homogeneous system. They are:

1. The equation of motion of barycentric flow (ignoring viscosity):

$$\frac{\partial \rho \mathbf{u}}{\partial t} = -\nabla p + \sum_i c_i \mathbf{X}_i - \nabla \cdot (\rho \mathbf{u}\mathbf{u}) \tag{1}$$

where i is taken over all chemical species including the membrane, $\mathbf{X}_i$ is the external force per particle (which may be electrical, gravitational, or in a particular case the external force exerted by the membrane clamps required to prevent membrane motion), and the local velocity $\mathbf{u}$ is defined by

$$\begin{aligned} \rho \mathbf{u} &= \sum \rho_i \mathbf{u}_i, \\ \rho &= \sum_i \rho_i. \end{aligned} \tag{2}$$

$\mathbf{u}\mathbf{u}$ is a dyadic product.

ρ_i is the density of each species, t is the time, p is the local equilibrium pressure, and c_i is the composition variable ($c_i = x_i/v$ where x_i is the mole fraction of component i, and v is the mean molecular volume). Note $\rho_i = c_i m_i$, where m_i is the mass per molecule of component i.

2. The equation of motion of each species separately:

$$\frac{\partial \rho_i \mathbf{u}_i}{\partial t} = -c_i \nabla \mu_i - \sum_{\beta \neq i} c_i c_\beta \xi_{i\beta} (\mathbf{u}_i - \mathbf{u}_\beta) + c_i \mathbf{X}_i - \nabla \cdot \rho_i (\mathbf{u}_i \mathbf{u} + \mathbf{u}\mathbf{u}_i - \mathbf{u}\mathbf{u}) \tag{3}$$

where μ is the local chemical potential, ξ is the friction coefficient (which is related to the diffusion coefficient).

3. The equation of continuity:

$$\nabla \cdot \rho \mathbf{u} = -\frac{\partial \rho}{\partial t}. \tag{4}$$

In the linearized approximation the equations in the membrane region (specialized for Teorell's problem) appear below.

1. The equation of the solute species:

$$0 = -c_s \nabla \mu_s - \sum_{\beta \neq s} c_s c_\beta \xi_{s\beta} (\mathbf{u}_s - \mathbf{u}_\beta) - c_s z_s \nabla \psi \tag{5}$$

where β is taken over all other species including the solvent and the membrane. The solute flux is constant, and z_s is the electrical charge per ion. ψ is the electrical potential.

2. The equation of motion of the membrane:

$$0 = -c_m \nabla \mu_m + \sum_\gamma c_m c_\gamma \xi_{m\gamma} \mathbf{u}_\gamma - c_m z_m \nabla \psi + \mathbf{F}_m \tag{6}$$

where γ is taken over all other species including the solvent and solute.

3. The equation of motion of barycentric flow becomes:

$$\frac{\partial \rho \mathbf{u}}{\partial t} = -\nabla p - \sum_i c_i z_i \nabla \psi + \mathbf{F}_m. \tag{7}$$

In Teorell's problem $\partial \rho \mathbf{u}/\partial t = \partial \rho_w \mathbf{u}_w/\partial t$ where w symbolizes water.

The equations for the aqueous system are similar except that gravitational forces operate, and there is no membrane species.

The equations were linearized by the standard perturbation method.[4] All the variables are expanded in a power series in ϵ (which is later taken to be unity) and all terms in ϵ^2 and higher powers are dropped for a first-order approximation. The variables are then substituted in the differential equations. Terms in ϵ^0 (order zero) are separated from those in ϵ^1 (first-order), and the differential equations are solved to each order separately. For our particular problem to 0th order the system has a steady-state pressure profile, constant current, and a constant uniform electrical field but no flow of solvent. We consider the first-order terms to be small and time-dependent. Other steady-state conditions could have been chosen for 0th order (e.g., constant flow of solvent), but those we have chosen yield simple equations which in turn yield more transparent results.

Equation (6) gives us the expression to use for $\mathbf{F}_m$ in equation (7). Equations (5) and (7) are used to solve for the perturbation value of c_s and finally equation (5) is used to solve for the perturbation value of $\nabla \psi$. The chemical potential is transformed by the relationship

$$\nabla_T \mu_i = \left(\frac{\partial \mu_i}{\partial c_i}\right)_{T,p} \nabla c_i + \left(\frac{\partial \mu_i}{\partial p}\right)_{T,c_i} \nabla p, \tag{8}$$

which, for simplicity, assumes ideal behavior. Note that $\partial \mu_i/\partial p$ is the partial molar volume $\bar{v}_i$ and $\partial \mu_i/\partial c_i$ is kT/c_i. With the values of $\nabla \psi$ and $\mathbf{F}_m$ substituted into equation (7) we can integrate the transformed equation (7) with respect to x across the membrane. The key differential equation to be integrated is

$$\nabla p_1 (1 - c_m \bar{v}_m - \sum_s c_{s0} \bar{v}_s) = -\frac{\partial \rho_w \mathbf{u}_w}{\partial t} + \sum_s kT \nabla c_{s1} - (\sum_s c_{s0} c_w \xi_{sw} + c_m c_w \xi_{mw}) \mathbf{u}_w, \tag{9}$$

where the subscript 0 denotes the 0th order term and the subscript 1 denotes the first-order term. This equation could have been obtained directly from the first-order equation for water and the Gibbs-Duhem relationship, but we have presented it differently because of the insight we gain thereby into the interdependence of the variables. We later utilize the fact that for isobaric one-dimensional flow

$$c_{s0} u_s = J_s = -\frac{D}{h} [c_{s0}(h) - c_{s0}(0)] - \frac{D z_s \partial \psi_0/\partial x}{kT} \frac{\int_o^h c_{s0} dx}{h} \tag{10}$$

where

$$\frac{kT}{D} = c_w \xi_{sw} + c_m \xi_{sm}$$

and ideality is assumed. We have also assumed that the friction coefficients ζ are independent of concentration.

The solutions must satisfy the following boundary conditions: (1) All flows must be continuous across the boundary. (2) The perturbation values of the concentration must be zero at the membrane boundaries since the external solutions are stirred. (3) The pressures must be continuous throughout the system. We seek solutions for laminar flow of the form $\mathbf{u}_w = -\nabla\phi$. These satisfy the equation of continuity if the system is assumed incompressible. We use a trial function of the form $\phi = Axe^{nt}$ which corresponds to spatially uniform velocity in the horizontal direction.

The equations are integrated with respect to x (one-dimensional problem) and the boundary conditions used to relate the solutions for the aqueous region to those of the membrane. The resulting relation is then differentiated with respect to time. The final form is used to solve for the value of n in $\exp(nt)$. The value of n gives us the stability condition.

The stability condition derived for a simple system involving flow of one ionic species (the case of co-ion exclusion) and with symmetrical geometry is

$$n = -R \pm \sqrt{R^2 - Q} \tag{11}$$

where

$$R = \frac{1}{2m_w}\left[\xi_{sw}\bar{c}_{s0}(m) + c_m\xi_{mw} - (1 - c_m\bar{v}_m)\xi_{sw}L\frac{\Delta c_{s0}}{h}\right],$$

$$Q = \frac{2}{h}(1 - c_m\bar{v}_m)\alpha g.$$

Here Δc_{s0} is the difference, c_{s0} (left compartment) $- c_{s0}$ (right compartment), h is the thickness of the membrane, α is a geometrical factor which becomes unity if the system has constant cross-sectional area, $\bar{c}_{s0}(m)$ is the average salt ion concentration in the membrane and is related to the current and concentrations through relation (10), and L is the distance of the electrode from the membrane. We note in particular that equation (10) reveals that threshold values exist for the current which determine the behavior of n.

Obviously, the above relationship can be real positive (corresponding to the onset of unstable turbulent flow), real negative (damped motion), purely oscillatory, or either damped or increasing oscillatory motion depending upon the values of the various quantities.

The analysis may be extended by considering functions of the form $\exp(-Kx + Ky + nt)$. The value of n will then be a function of K, and the allowable values of K will be restricted to those that satisfy boundary condition 2. We also require the vertical velocity to be zero at the boundary between membrane and wall. Thus, the value of the velocity potential will be a superposition of many functions which causes the periodic motion to be a distorted wave form where the wave form may change with time.

When viscosity is included in the equations of motion, the effect is to damp out the contribution of the higher-frequency modes.[5] The introduction of more than one species makes the result more complicated (and flexible) but does not change

the essence of the physical results. The effect of displacement current, partition coefficients, and other equilibria and boundary (sheath)[6] problems can be treated in a more sophisticated derivation.

Although the derivation determines the time dependence of the velocity potential, the time dependence of $\Delta\psi$ and Δp may be determined through the use of the fundamental set of first-order equations. Thus, we may determine the response of these variables.

In examining the stability condition we note that for our particular problem $\nabla\psi_0$ (through $\bar{c}_{s0}$) functions like a stimulus condition for periodic response. From (8) and (5) we note that ∇c and ∇p play a role similar to that of $\nabla\psi$. Hence, we have a mechanism which is responsive to 0th order "inputs" of constant Δc, Δp, or $\Delta\psi$ across the membrane. In the more general theory,[3,7] $\nabla \ln T$ is also similar to the other variables. Thus, we have a single mechanism which may explain nerve response to chemical, mechanical, electrical, and thermal stimuli!

We are planning to demonstrate that other types of hydrodynamic stability conditions can arise from other model systems involving membranes. One of these model systems will resemble the nerve more closely. In these systems, surface forces rather than gravity are expected to play the dominant role. Membrane motion will be permitted. The theory may also be brought to bear upon several curious observations in paper chromatography.[8]

Although Teorell's membrane oscillator is a very specific model, the fact that hydrodynamic relationships in the membrane can cause periodic behavior in this model suggests that the hydrodynamic mechanism may underlie many of the oscillatory biological processes which involve membranes. At any rate, now all the techniques and experience of plasma physics can be brought to bear upon the problem.

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*A THEORY OF ENERGY TRANSFER IN THE PHOTOSYNTHETIC UNIT**

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1. *Introduction.*—Recent developments have encouraged us to propose a theory of energy transfer in the photosynthetic unit. The PSU[1] was first postulated as a physiological unit by Emerson and Arnold in 1932.[2] This concept, based on flashing-light studies, now seems firmly established.[3] More recently, attempts have been made to identify the PSU as a definite cellular substructure. Park and Pon have isolated particulate fragments,[4] called quantasomes, from the lamellae of spinach chloroplasts, which can efficiently perform the light reactions of photosynthesis. It has been proposed that quantasomes may be the PSU's of algae and higher green plants.

The role of the PSU, as a unit for transferring energy absorbed anywhere in an aggregate of pigment molecules to a single "active" site, has been discussed by many authors.[5] Following Franck and Teller's initial discussion[6] of physical mechanisms for energy transfer in the PSU. two schools of thought emerged. Förster[7] proposed that energy is transferred between adjacent pigment molecules via weak (dipole-dipole) resonance interactions. His proposal was criticized by Franck and Livingston,[8] but the idea of resonance transfer was revived by Duysens,[9] who studied sensitized fluorescence *in vivo*.

Various strong interaction mechanisms have also been proposed. These include conduction bands, free excitons, and electron or hole diffusion.[5] All of these mechanisms require either electron-orbital overlap or electromagnetic coupling frequencies higher than molecular relaxation rates. Rabinowitch has pointed out that spectroscopic evidence is against the existence of such crystalline or quasi-crystalline states of chlorophyll *in vivo*.[10] We consider it unlikely, therefore, that strong coupling mechanisms play an important role in energy transfer in the PSU. We will show that a theory based on the incoherent limit[11] of weak coupling leads to a working model of the PSU.

Our interaction strengths are taken from Förster's weak coupling case.[12] However, as noted by Rabinowitch[10] and by Lumry and Spikes,[13] a simple pairwise interaction model is inadequate to deal with the PSU, because the "jumping time" (making extreme use of the localized picture) depends on interactions with many neighbors. The transfer rate increases with the number of nearest-neighbor pathways of energy transfer,[11] or branchings, B. Neglecting interactions with nonnearest neighbors, the relation between actual transfer time (reciprocal of rate) t_B and pairwise transfer time t_1 is

$$t_B = t_1/B. \tag{1}$$

Since B increases in general with D, the number of dimensions in which the molecules are arrayed, the transfer rate is greatest, for given t_1, when $D = 3$. The concept that Chl is arrayed in monomolecular layers ($D = 2$), derived from elec-

tron microscope observations of chloroplast lamellae,[14] need not be retained in light of the quantasomes. We consider both $D = 3$ and $D = 2$ cases.

2. *Theory.*—We view the PSU as an aggregate of pigment molecules and an active center, the trap, bound in a lipid-protein environment.[13] We consider only algae and higher green plants in which Chl-a is the most abundant pigment. Recent evidence suggests that there may be two pigment systems absorbing quanta for two light reactions *in vivo*.[15] However, investigators affirm that energy absorbed by any pigment is only usefully trapped for photosynthesis through excited states of Chl-a.[15, 26] Thus, we construct a theory of energy transfer through the bulk Chl-a (of either pigment system) to the trap, which is independent of whether the Chl-a is excited by accessory pigments or directly by the radiation field. In addition, we assume that each PSU transfers energy only to a uniquely associated trap, as is indicated by experiments of Park and Pon[4] and of Butler and Baker.[16]

The apparent red-shift[17, 18] of the trap absorption band with respect to that of the bulk Chl-a justifies the assumption that energy transfer to the trap be highly irreversible. For simplicity, we treat the trap as a perfect energy sink, when the trap is open (see *Discussion*). If the bulk Chl-a molecules have equal probabilities for initial excitation and a uniform spatial distribution (at least for PSU-ensemble averages), the trap is most efficiently located with regard to energy transfer at the geometrical center of the bulk Chl array. However, if the trap is located near an extremity of the array, the mean trapping time is only increased by about a factor of two. Results are given for a central trap.

Because of the inverse sixth power dependence on distance of dipole-dipole interaction rates, regular spacing of the bulk Chl-a molecules is more efficient for energy transfer than random spacing. The high concentration of Chl-a in the PSU prevents large spacing fluctuations. In addition, Butler and Baker's recent finding that the ratios of pigment concentrations in quantasomes are the same as in intact chloroplasts[16] suggests that pigment molecules are bound in the PSU. Thus, it is likely that the spacing fluctuations are quite small. Average fluctuations on the order of 10% reduce the transfer rate only by about 15% from the regular spacing rate. For simplicity, we use a mathematical model based on regular spacing.

As noted in reference 11, the equations for resonance transfer in the incoherent limit are

$$\left(\frac{d}{dt} + \frac{1}{\tau}\right)\rho_k = \sum_{l=1}^{N} F_{kl}(\rho_l - \rho_k), \tag{2}$$

$k = 1, \ldots N$. Here, ρ_k is the average excitation amplitude squared of the k^{th} molecule, N the number of molecules in the aggregate, F_{kl} the pairwise transfer rate between molecules k and l, and τ the excitation decay time constant due to radiation and ordinary quenching processes (degradation to heat).

Since roughly 80% of the dipole-dipole interaction energy comes from nearest-neighbor interactions, we neglect all other interactions for simplicity; inclusion of the remaining terms leads to slightly higher transfer rates. Equations (2) then reduce to a single second-order difference equation, which in turn is very well approximated by a diffusion equation,[19]

$$\left(\frac{\partial}{\partial t} + \frac{1}{\tau}\right)\rho(\mathbf{r},t) = \Lambda\nabla^2\rho(\mathbf{r},t), \tag{3}$$

where ρ as a function of the continuous variable $\mathbf{r}$ is an excitation probability density, $\mathbf{r}$ is a vector defining coordinates in two or three dimensions, ∇^2 the Laplacian operator, and Λ the diffusion constant.

The probability, $P(t)$, that the bulk Chl-a of the PSU, as a whole, is excited at time t is

$$P(t) = \int_{\text{PSU}} \rho(\mathbf{r},t)dV, \tag{4}$$

where dV is an area or volume element. The mean de-excitation time of the PSU is

$$\bar{t} = \int_0^\infty t\left(\frac{dP}{dt}\right)dt \Big/ \int_0^\infty \left(\frac{dP}{dt}\right) dt. \tag{5}$$

We normalize to $P(0) = 1$; then, since $P(\infty) = 0$,

$$\bar{t} = \int_0^\infty P(t)dt. \tag{6}$$

Now,

$$\frac{dP}{dt} = -\left(\frac{dP_T}{dt} + \frac{dP_F}{dt} + \frac{dP_Q}{dt}\right), \tag{7}$$

where dP_T/dt is the rate of trapping, dP_F/dt that of fluorescence, and dP_Q/dt that of quenching. Since the latter two are much smaller than dP_T/dt (see *Discussion*), they can be neglected in (5), and thus $\bar{t} = \bar{t}_T$, the mean trapping time.

In terms of the random walk description, the mean number of jumps to reach the trap, n, is related to the mean trapping time by

$$\bar{t}_T = nt_B, \tag{8}$$

where t_B is given by (1). Thus, $\bar{t}_T$ may be calculated directly from the solution of the diffusion equation, or from (8) if n is known. We rely primarily on the former for the $D = 3$ case. For $D = 2$, recent calculations have given a value for n, which we use below.

$D = 3$ case: The diffusion equation (3) may be solved when appropriate boundary and initial conditions are prescribed. For mathematical simplicity, we approximate the ellipsoidal quantasome by a sphere of radius a, the trap by a small concentric spherical cavity of radius b. It is then most convenient to use a system of spherical coordinates, with $r = |\mathbf{r}|$, θ, and ϕ. The statement that there be no energy transfer between PSU's becomes mathematically,

$$(\partial\rho/\partial r)_{r=a} = 0; \tag{9a}$$

that the trap be a perfect sink, by analogy to the theory of heat conduction,[20]

$$[\rho(\mathbf{r},t)]_{r=b} = 0. \tag{9b}$$

Since we are only interested in average properties of ensembles of PSU's, we may treat the problem as if the excitation energy were initially uniformly delocalized over the PSU.[11] Thus, $\rho(\mathbf{r},0)$ is independent of $\mathbf{r}$, and because $P(0) = 1$, equation (4) gives

$$\rho(\mathbf{r},0) = 3/4\pi a^3(1 - \epsilon^3), \tag{10}$$

where $\epsilon = b/a$. The problem is then independent of θ and ϕ, and equation (3) simplifies to

$$\left(\frac{\partial}{\partial t} + \frac{1}{\tau}\right)\rho = \frac{\Lambda}{r^2}\frac{\partial}{\partial r}\left(r^2\frac{\partial \rho}{\partial r}\right). \tag{3'}$$

Equation (3') together with conditions (9) and (10) may be solved exactly by standard techniques[20] to give

$$\rho(r,t) = \frac{3\epsilon}{2\pi a^3(1-\epsilon^3)}\sum_{i=0}^{\infty}\frac{(1+\xi_i^2)\sin\left[\xi_i\left(\frac{r}{a}-\epsilon\right)\right]}{\xi_i[\xi_i^2(1-\epsilon)-\epsilon]r/a}e^{-\beta_i t}, \tag{11}$$

where $\beta_i = \tau^{-1} + (\Lambda/a^2)\xi_i^2$, and the ξ_i are all of the positive solutions to the eigenvalue equation

$$\tan[\xi_i(1-\epsilon)] = \xi_i, \tag{12}$$

ordered such that $0 < \xi_0 < \xi_1 < \ldots$.

The expression (11) for $\rho(r,t)$ is an expansion in transient diffusion modes. The function $P(t)$ derived from (4) is thus an infinite series of exponential decays. The relative coefficient of the fundamental decay mode is, however, about 95%. The theory thus predicts that the excitation decay is purely exponential, except for very small, rapidly decaying, initial transients.

For $\epsilon = 0.20$ (see *Discussion*), numerical solution of (12) gives $\xi_0^2 = 0.90$. Then, using (4), (6), and (11), and neglecting τ (see *Discussion*), the mean trapping time is

$$\bar{t}_T = 1.1\ a^2/\Lambda. \tag{13}$$

The diffusion constant is given by

$$\Lambda = \bar{R}^2/t_1, \tag{14}$$

where $\bar{R}$ is the mean separation of adjacent Chl-a molecules. For the spherical model, we have, approximately,

$$(a/\bar{R})^2 = (3N/4\pi)^{2/3}. \tag{15}$$

From the preceding, we see that

$$\bar{t}_T = 0.4N^{2/3}t_1, \tag{16}$$

and thus that,

$$n = 0.4BN^{2/3}. \tag{17}$$

D = 2 case: The solution of the random walk problem for a square lattice of N sites is given approximately by[21]

$$n = 1.3N - N^{1/2} \tag{18}$$

where it is assumed that the trap occupies one lattice site at the center of the array. We thus have immediately that

$$\bar{t}_T = (1.3N - N^{1/2})t_B. \tag{19}$$

3. *Results and Discussion.*—In order to make predictions from the theory, it is necessary to estimate values of parameters which are not well known.

Number and spacing of Chl-a molecules: Emerson and Arnold found that under conditions of saturation about 2000 Chl-a molecules are required for each CO_2 reduced in Chlorella.[2] If the quantum requirement for CO_2 reduction of each pigment system is about 4,[15] there is a total of some 500 Chl-a's available to both pigment systems, assuming the two together form a biologically operational unit. It is believed that most of the Chl-a's are associated with pigment system 1 (the long-wavelength absorbing system).[15] Thus, we take $N \simeq 400$ for system 1.

We base our estimates of $\bar{R}$ on the quantasome, described as an oblate spheroid, 100 Å by 200 Å.[4] If the sphere of the $D = 3$ model is assumed to have the volume of the quantasome, $a = 79$ Å, and hence, $\bar{R} = 17$ Å. If the square array of the $D = 2$ model is assumed to have an area equal to half[22] the surface area of the quantasome, $\bar{R} = 11$ Å.

Pairwise transfer time: If we write (for dipole-dipole interactions)

$$t_1 = \tau_0(\bar{R}/R_0)^6, \tag{20}$$

the parameter R_0 may be calculated from Förster's theory if the natural fluorescence lifetime τ_0, the overlap of absorption and fluorescence bands, the relative orientation of the two molecules, and the solvent refractive index μ are known.[23, 24] Förster gives a value of 80 Å for R_0 for Chl-a at low concentration in ethyl ether.[23] However, he assumed a value of 30 nsec for τ_0, whereas more recent calculations give $\tau_0 = 15$ nsec.[25] Based on the latter value of τ_0, $R_0 = 71$ Å for Chl-a in ethyl ether.

We may estimate the value of R_0 *in vivo*. The greater width of the Chl-a (red) absorption band *in vivo* suggests that the spectral overlap there may be different from what it is in solution. However, it now appears that the greater width is due to two forms of Chl-a (possibly belonging to the two pigment systems).[26] Without further specific information, we assume that no correction to R_0 of the solution is required for differing spectral overlap in the plant. Since orientations *in vivo* are also unknown, we accept a random directional distribution as was used in the solution calculation. The refractive index of lipid-like materials, however, is $\sim$1.5, whereas $\mu = 1.35$ for ethyl ether. Thus, as a rough estimate, we find $R_0 = 68$ Å for Chl-a *in vivo*.

With the above values of τ_0, R_0, and $\bar{R}$, we find, for system 1, the values of t_1, t_B, and Λ given in Table 1. We use $B = 6$ for $D = 3$, and $B = 4$ for $D = 2$. The diffusion constants are about 10^3 greater than those typical of diffusion of aqueous solutions into water. It appears highly unlikely, therefore, that primary energy transfer could be effected as efficiently by diffusion of high-energy intermediates[10] as by resonance transfer.

Trap parameters: When the trap is open (or active), the mean observable fluorescence lifetime, $\bar{t}_F$, is equal to $\bar{t}_T$. When the trap is closed, however, $\bar{t}_F = \tau$, which follows from (3) when the boundary condition (9b) is replaced by $(\partial\rho/\partial r)_{r=b} = 0$. Since the *primary* process of photosynthesis is thought to be more than 90 per cent efficient, it follows that $\tau \gg \bar{t}_T$. Thus, we have neglected τ in the calculations.

The diffusion model allows us to consider a range of trap sizes in the $D = 3$ case. We assume that the trap occupies a single lattice site, and thus take b to be about

15 Å, which gives $\epsilon = 0.20$. The trapping volume is then large enough to contain a number of chlorophyll molecules, and even the quantatrope of Sauer and Calvin.[27]

System 1 results: The results for n, $\bar{l}_T$, and $\varphi_F = \bar{l}_T/\tau_0$ are shown in Table 1. The $D = 2$ trapping time and fluorescence yield are about half the $D = 3$ result. However, the $D = 2$ actual transfer rate, t_B^{-1}, is greater than intramolecular relaxation rates ν_m, which are $\sim 10^{12}$ to 10^{13} sec^{-1}. Thus, the weak-coupling theory is not valid in the $D = 2$ case, and transfer rates may be even higher than calculated. As noted earlier, spectroscopic evidence rules against strong interactions in the PSU; therefore, we feel that a two-dimensional model is inappropriate for energy transfer in the PSU.

Even though t_B^{-1} for $D = 3$ is comparable to ν_m, the lack of spectroscopic evidence to the contrary indicates that the weak-coupling limit is still applicable as an approximation. The $D = 3$ results for $\bar{l}_T$ and φ_F should therefore be regarded as upper limits.

TABLE 1

SYSTEM 1 RESULTS

D	t_1 psec	t_B psec	Λ cm^2/sec	n	$\bar{l}_T$ psec	φ_F, per cent
2	0.3	0.07	0.040	500	36	0.24
3	4	0.7	0.007	130	86	0.57

System 2 (short-wavelength absorbing system): If a second pigment system, containing fewer Chl-a molecules, exists, it may be expected to make an additional contribution to the fluorescence yield. As a possibility, suppose 100 Chl-a's and a trap were arrayed within a quantasome-equivalent sphere; $\bar{l}_T$ would be about 0.4 nsec, and φ_F about 3 per cent. The *average* yield for both systems together would then be about 1 per cent. Even if both systems have identical fluorescence spectra, a fast-pulse experiment having a resolving time $\leqq 0.1$ nsec would be able to separate the two contributions.

Comparison with experiment: In addition to rapid emission by the bulk Chl-a, presumably at a wavelength around 680 mμ, there are at least two other sources of luminescence *in vivo*. These are rapid emission (supposedly by the trap itself) at around 730 mμ, with a measured lifetime of 3.1 nsec;[17] and slow emission, or delayed light, by both pigment systems predominantly at the shorter wavelength ($\sim$680 mμ).[28] It has recently been suggested that the delayed light extends to times as short as 10^{-6} sec or less, and that the integrated delayed light emission, because of its slow decay, may make a larger contribution to the integrated fluorescence yield than the fast emission components of comparable wavelength.[29]

The 1.7 nsec lifetime of Chl-a *in vivo* measured by Brody and Rabinowitch,[25] the 0.7 nsec lifetime obtained by Butler and Norris,[17] and the 2.7 per cent fluorescence yield of Latimer *et al.*[30] must be re-evaluated in light of these recent developments. Neither the Brody nor the Butler experiments discriminated between the 730 mμ and 680 mμ emissions, and may have given an average value. The Latimer experiment integrated both fast and slow emissions. Hence, the small values of φ_F and $\bar{l}_T$ calculated here are entirely compatible with available experimental evidence.

Summary.—A mathematical model of energy transfer in the photosynthetic unit, based on weak interactions, is developed. Predictions of mean trapping time are

derived from the estimated (dipole-dipole) interaction strength. A diffusion equation, derived directly from the delocalized picture,[11] is used in the calculations.

The trapping times calculated here are surprisingly short compared to previous estimates based on a random-walk description. It appears that two points were overlooked in the latter. First, the number of "jumps" depends sensitively on the number of dimensions. Second, a simple pairwise interaction is inadequate because the transfer rate is proportional to the number of nearest neighbors. These points are accounted for automatically by the diffusion treatment.

Estimates of transfer times, based on the dimensions of the quantasome, are given. Using these, explicit results for fluorescence lifetime and yield are calculated for the long-wavelength absorbing pigment system. The fluorescence lifetime of the short-wavelength system may be somewhat ($\sim$4 times) longer. The compatibility of the short calculated lifetimes ($\sim 10^{-10}$ sec) with known experimental results is demonstrated.

Both two- and three-dimensional arrays of chlorophyll-a molecules are considered. Experimental evidence favors the latter.

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[1] Abbreviations: PSU = photosynthetic unit; Chl = chlorophyll; nsec = nanosecond (10^{-9} sec); psec = picosecond (10^{-12} sec); a bar ($^-$) is used to denote averages.

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POLYRIBOSOME FORMATION AND HEMOGLOBIN SYNTHESIS

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Polyribosomes have been shown to be the major site of protein synthesis in reticulocytes,[1-3] liver,[4] HeLa cells,[5] and *E. coli*.[6] It seems likely that polyribosomes are clusters of ribosomes which are held together by messenger RNA.[1] This has led to the hypothesis that, during protein synthesis, single ribosomes attach to one end of the polyribosome and start the synthesis of a peptide chain. Chain growth proceeds as the ribosome moves along the messenger RNA, and finally completion and release of the polypeptide chain occurs when the ribosome detaches from the cluster at the end of the messenger.[3, 6-8] Earlier evidence for such a mechanism has been indirect. The formation of polyribosomes in the presence of various types of natural and synthetic messenger polynucleotides has been demonstrated.[5, 6, 9, 10] The breakdown of polyribosomes during protein synthesis in cell-free systems yielding single ribosomes with labeled nascent protein is consistent with such a mechanism.[6, 8] We have reported that 80S ribosomes were active in a cell-free system and joined the polyribosome clusters during hemoglobin synthesis and also that polyribosomes broke down during protein synthesis.[11] More detailed studies showed the strict dependence of polyribosome breakdown on protein synthesis and the orderly disappearance of the various sizes of polyribosomes expected for the mechanism postulated above.[12] The formation of polyribosomes using H^3-labeled ribosomes from HeLa cells has been reported.[13] Here, we demonstrate the attachment of P^{32}-labeled 80S ribosomes to polyribosomes correlated with initiation of polypeptide chain synthesis, and detachment of 80S ribosomes correlated with polypeptide chain completion and release. These results provide strong support for the hypothesis described above.

Materials and Methods.—The methods and enzyme preparations used were those described previously unless otherwise noted.[12] To prepare P^{32}-labeled ribosomes, rabbits were given the usual four daily injections of phenylhydrazine at a dosage of 0.9 ml of 2.5% phenylhydrazine per 5 lb. On the evening of the fifth day and the morning and evening of the sixth (36, 24, and 12 hr before bleeding), each rabbit received 1 mC of carrier-free P^{32} by intravenous injection (3 mC total). Carrier-free P^{32} was obtained from the Oak Ridge National Laboratories and was diluted to contain 2 mC/ml at pH 7.4 in 0.01 *N* Tris, 0.9% in NaCl. The rabbits were bled by heart puncture on the morning of the seventh day, and 1X ribosomes (pelleted once) were prepared

as usual from their blood.[14] Ribosomes at the 1X stage prepared from these cells contained at least 40,000 cpm P^{32}/mg when counted in the Packard Tri-Carb scintillation counter under standard conditions. When assayed in the complete cell-free system, the P^{32}-labeled ribosomes incorporated from 5 to 8 mμmoles of total amino acid into hemoglobin per mg of ribosomes in 60 min.

80S ribosomes were prepared from 1X ribosomes by sucrose gradient fractionation.[12] In a typical experiment, 1.2 ml (24 mg) of P^{32}-labeled 1X ribosomes were layered onto each of two gradient tubes. After centrifugation, 30 fractions (1 ml each) were collected from each gradient, and the two tubes containing the peak portion of the 80S ribosomes from each gradient were pooled (tubes 20 and 21). In this experiment, 4 ml containing 0.92 mg/ml of 80S ribosomes were recovered.

"Derived" ribosomes were prepared by incubating polyribosomes isolated from sucrose gradients (pooled tubes 1–15 of the standard 30-tube gradient) in the complete cell-free system for 60 min. Polyribosome breakdown was essentially complete in this time as judged both by analytical ultracentrifugation and by sucrose gradient fractionation. The derived 80S ribosomes were concentrated for use by sedimenting at 150,000 × *g* for 1 hr and resuspending the pellet as in the 1X ribosome preparation.

Polyribosome formation was assayed by incubating 6 mg of unlabeled 1X ribosomes plus 0.4 mg of P^{32}-labeled 80S ribosomes in 1.4 ml of incubation mixture using "purified" enzyme.[12] After incubation for the desired time, the mixture was cooled in an ice bath and 1.0 ml was layered onto a sucrose gradient tube at 0°. A 0.2-ml aliquot of the remaining mixture was added to 0.6 ml of solution containing 9 mg of casein and precipitated with 5 per cent TCA. The sample was taken through the complete washing procedure. Radioactivity was determined in the Packard Tri-Carb scintillation counter using window settings suitable for counting C^{14} and P^{32} simultaneously. Total incorporation of amino acids into hemoglobin was calculated from C^{14}-valine incorporation with the use of absolute C^{14}-standards. Incorporation of P^{32}-80S ribosomes into polyribosomes was measured after gradient fractionation, either by collecting sixty 0.5-ml fractions directly into Tri-Carb vials, or by collecting thirty 1-ml samples into tubes. In the latter case, the optical density at 260 mμ of a fraction was determined on a diluted aliquot, and 0.5 ml of the remainder was added to a Tri-Carb vial. Before counting the fractions, counting fluid was added to the vials directly without addition of the sodium hydroxide or dioxane generally used.[15] P^{32}-samples were corrected for decay. Control experiments showed that at least 90% of the P^{32} in ribosomes was in RNA which sedimented in a sucrose gradient as expected.

The preparation of ribosomes labeled with H^3-leucine in the whole cell has been described.[16] The per cent of N-terminal C^{14}-valine in hemoglobin was determined by the dinitrofluorobenzene method of Bishop *et al.*[16]

Results.—Polyribosome formation and breakdown: Attachment of 80S ribosomes to polyribosomes was observed when P^{32}-labeled 80S ribosomes plus unlabeled 1X ribosomes (see *Methods*) were incubated in the complete system for amino acid incorporation (Fig. 1). Controls incubated at 0° in the complete system, or at 37° in the absence of energy, were similar to each other and to labeled 80S ribosomes alone (Table 1). These results demonstrate that the attachment of 80S ribosomes to polyribosomes is an energy-dependent reaction, presumably reflecting a requirement for peptide bond synthesis. The increase of P^{32} in the polyribosome region is similar to the decrease in labeled 80S ribosomes, indicating that there is little nonspecific loss of labeled 80S ribosomes during the incubation or on the sucrose gradient.

The amount of attachment may be calculated quantitatively, as follows. Approximately 4.25 mg of unlabeled 1X ribosomes, which contained 1.35 mg of 80S ribosomes, plus 0.26 mg of P^{32}-labeled 80S ribosomes were placed on the gradient after incubation in the complete system for 5 min. The total number of P^{32} counts recovered was 12,000 counts/min. The number of P^{32} counts attached to

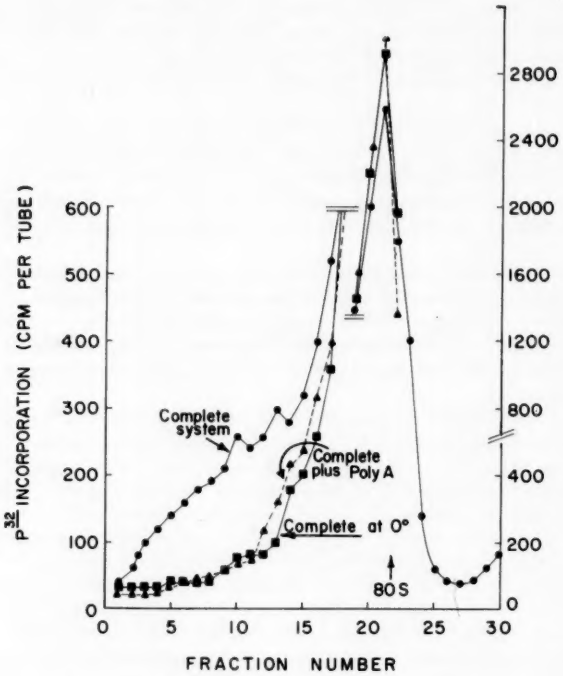

FIG. 1.—Attachment of P^{32}-labeled 80S ribosomes to polyribosomes. P^{32}-labeled 80S ribosomes plus 1X unlabeled ribosomes were incubated in the complete cell-free system for 5 min and then fractionated on a sucrose gradient (see *Methods*). The data are given as counts per min of P^{32} in each tube. Data for tubes 0–18 are given on the left ordinate, tubes 19–30 on the right ordinate.

the polyribosome peak was 1,800, based on the difference between the samples incubated at 37° and 0° (Fig. 1). Thus, approximately 15 per cent of the 80S ribosomes, corresponding to 0.24 mg, was attached to 2.90 mg of polyribosomes. Since the average polyribosome contains four ribosomes, based on the per cent composition of polyribosomes reported previously,[12] it appears that 35 per cent of the poly-

TABLE 1

ATTACHMENT OF P^{32}-LABELED 80S RIBOSOMES TO POLYRIBOSOMES

Incubation conditions	P^{32} in polyribosomes counts per minute
Complete system at 0°	245
Complete, minus energy	330
Complete	1610
Complete plus poly A	710
Complete, derived 80S ribosomes	1460
Above incubated at 0°	0

Samples of P^{32}-labeled 80S ribosomes plus unlabeled 1 X ribosomes were incubated in the complete amino acid incorporating system for 5 min (see *Methods*) and then fractionated on a sucrose density gradient. The total P^{32} attached in tubes 0–13 was considered to be the polyribosome peak. Labeled 80S ribosomes alone gave 690 counts in tubes 0–13 and this has been subtracted from the values given. In "minus energy," the ATP, GTP, creatine phosphate, and creatine kinase were omitted, and hexokinase plus glucose added to trap endogenous ATP. Poly A was added at a concentration of 150 μg per 1.4 ml of incubation mixture. Derived P^{32}-labeled 80S ribosomes were prepared by incubating labeled polyribosomes in the complete system for 60 min,

ribosomes have attached a new ribosome during the 5-min incubation on the average. This estimate is probably low (see *Discussion*). Another point of interest is that ribosome attachment occurs all along the gradient into all the classes of polyribosomes in a 5-min incubation (Fig. 1), and also in a 3-min incubation (unpublished data).

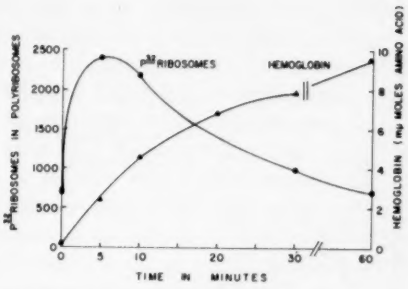

FIG. 2.—Time course of attachment of P^{32}-labeled 80S ribosomes to polyribosomes. The samples were incubated as described in Fig. 1 for various times and fractionated on a sucrose gradient. Each point represents the sum of the P^{32} found in tubes 0–13 (the polyribosome region). Data are given on the left ordinate. For comparison, the time course of amino acid incorporation into hemoglobin is shown (right ordinate). Data here is given as mμmoles of total amino acid incorporation per mg of ribosomes.

The time course of attachment is very rapid compared to hemoglobin synthesis (Fig. 2) so that maximum attachment is attained in about 5 min, and by 10 min some newly attached ribosomes have already left the polyribosome. Almost all of the P^{32}-ribosomes which attach leave the polyribosomes by 40–60 min, which is when hemoglobin synthesis also stops. According to the hypothesis proposed, the detachment of these P^{32}-ribosomes should correspond to the release of globin chains synthesized *de novo* in the cell-free system, while the detachment of ribosomes *originally* in polyribosomes should produce chains which are only completed in the cell-free system, i.e., the N-terminal region is not labeled.

A very striking inhibition of P^{32}-ribosome attachment to polyribosomes was observed when poly A was added (Fig. 1), confirming our previous hypothesis[12] of the mechanism by which poly A inhibited hemoglobin synthesis. The inhibition of P^{32}-ribosome attachment by poly A varied from 80 to 95 per cent in several experiments at the concentration used (Table 1). Derived ribosomes, produced from polyribosomes by incubation in the complete cell-free system, were nearly as active in attachment to polyribosomes (Table 1) as the original 80S ribosomes found after cell lysis.

Kinetics of hemoglobin synthesis and release: In order to study formation of the polypeptide chain, ribosomes were labeled in the intact cell with H^3-leucine. These labeled ribosomes were incubated in the cell-free system with C^{14}-leucine, with and without poly A. Almost all of the H^3-leucine is in peptide chains in the *polyribosomes*,[1, 2] and this has been confirmed in our own studies (unpublished data). Thus, the loss of the H^3-leucine from ribosomes into hemoglobin (Fig. 3*A*) represents the completion of already started polypeptide chains. This was not inhibited by poly A. Since polyribosome breakdown also was not inhibited by poly A,[12] this result provides the link showing that polyribosome breakdown involves the completion of already started chains. That is, ribosomes in the polyribosome have already begun the synthesis of the peptide chain. This result is consistent also with the data in Figure 1 showing that ribosome attachment requires energy, suggesting that peptide bond formation is needed to form the polyribosome.

The cell-free incorporation (Fig. 3*B*) of the same ribosomes, on the other hand, was

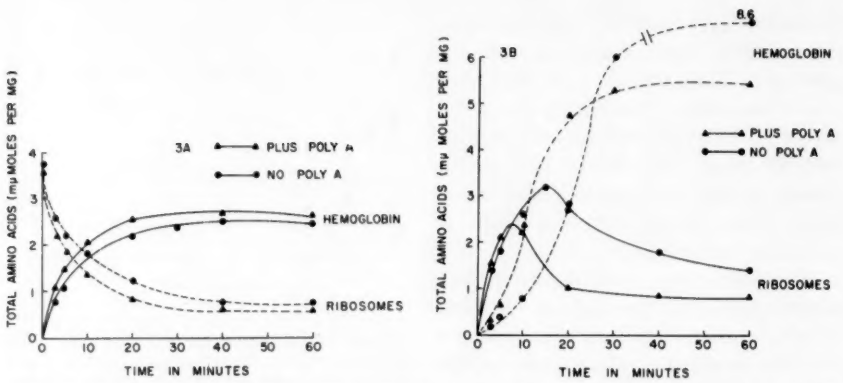

FIG. 3.—(*A*) Time course of hemoglobin synthesis from ribosomes labeled in the intact cell. Ribosomes labeled in the intact cell with H^3-leucine were incubated in the complete cell-free system with C^{14}-leucine. The dashed lines show the loss of H^3-leucine from the labeled ribosomes, and the solid lines the appearance of H^3-leucine in hemoglobin.

(*B*) Time course of incorporation in the cell-free system. This shows the cell-free incorporation of C^{14}-leucine using the same ribosomes as in (*A*). The dashed lines show incorporation into hemoglobin; the solid lines show incorporation into ribosomes. Data in both graphs are given as mμmoles of total amino acid incorporation per mg of ribosomes used.

partially inhibited by poly A. The incorporation into ribosomes showed the same initial rate with or without poly A, showing that the rate of peptide bond formation is not inhibited directly. However, incorporation into ribosomes ceased earlier in the presence of poly A. The difference in incorporation into ribosomes with and without poly A should represent the failure of new ribosome attachment in the presence of poly A. The time course of the inhibition and the amount of inhibition is generally consistent with this. The inhibition of hemoglobin synthesis by poly A was approximately 35 per cent (from 8.6 to 5.5 mμmoles, Fig. 3*B*). This amount of inhibition is the expected amount if *de novo* chain synthesis were prevented. The amount of *de novo* synthesis may be calculated from the N-terminal C^{14}-valine data (Table 2). The N-terminal C^{14}-valine found after 60 min was 3.4 per cent. Since uniformly labeled hemoglobin gives 8.3 per cent, then 40 per cent of the radioactivity is in chains synthesized *de novo*. The inhibition of *incorporation* by poly A is therefore consistent with the idea that few, if any, newly synthesized chains are made when poly A is present. This is confirmed by the inhibition of the appearance of N-ter-

TABLE 2

N-TERMINAL C^{14}-VALINE IN HEMOGLOBIN SYNTHESIZED IN THE CELL-FREE SYSTEM

Incubation conditions	Total Amino Acid Incorporation Ribosomes mμmolhs	Hemoglobin mμmoles	N-terminal C^{14}-valine per cent
Complete system, 10 min	1.9	2.1	0.6
Above, plus poly A	1.6	2.5	0.1
Complete system, 60 min	0.9	8.3	3.4
Above, plus poly A	0.7	5.7	1.0

Samples were incubated in the complete system with C^{14}-valine for the indicated times. Poly A was added at a concentration of 150 μg per assay. The ribosomes and supernatant were separated by centrifugation, and the incorporation in each was determined. Data are given as mμmoles of total amino acid incorporated per mg of ribosomes used. The amount of C^{14}-valine in N-terminal position in the supernatant protein was determined as described (see *Methods*). Uniformly labeled hemoglobin prepared by incubation in the intact cell gave the expected value of 8.3 per cent N-terminal C^{14}-valine. Hemoglobin labeled with C^{14}-leucine in the cell-free system gave 0.25 per cent N-terminal radioactivity which is the expected blank value and has been subtracted from the values given.

minal C^{14}-valine (indicative of *de novo* synthesis) in the presence of poly A (Table 2). The percentage of C^{14}-valine in N-terminal position after 60 min of incubation was reduced from 3.4 per cent to 1.0 per cent, while incorporation into hemoglobin was reduced from 8.3 to 5.7 mμmoles. Thus, the inhibition of *de novo* chain synthesis was the major effect of poly A by this criterion also. In this experiment (Table 2), the inhibition of incorporation by poly A was less than usual, and this probably accounts for the small amount of *de novo* chain synthesis which still occurred in the presence of poly A.

Discussion.—The discovery of polyribosomes as the site of protein synthesis led to a model in which a single ribosome attaches to one end of the linear cluster and initiates polypeptide chain synthesis, then moves along the messenger RNA strand while the polypeptide chain is synthesized, and then drops off the other end when the polypeptide chain is completed.[3, 6, 8] These studies and those of Goodman and Rich[13] are the first to show attachment of single ribosomes to natural polyribosomes during protein synthesis. The breakdown of polyribosomes in a cell-free system during protein synthesis[8, 12, 13] is consistent with this mechanism. However, the critical evidence reported here supporting this hypothesis is the demonstration that the polypeptide chain is started when single ribosomes attach to polyribosomes, and the peptide chain is completed and released when the single ribosome detaches. This demonstration depended on the finding that 1X ribosomes were capable of *de novo* chain synthesis in the cell-free system based on the incorporation of N-terminal C^{14}-valine. The 80S ribosomes were active, but attached to polyribosomes during hemoglobin synthesis, suggesting a correlation between ribosome attachment and *de novo* synthesis. In contrast, 2X ribosomes (pelleted twice) showed active 80S ribosomes which did not attach to polyribosomes and showed little *de novo* chain synthesis.[11]

The use of P^{32}-labeled ribosomes in the present studies in conjunction with the separation of chain completion from *de novo* synthesis made possible a direct test of this hypothesis. Firstly, the kinetics of new chain formation (*de novo* synthesis) are consistent with the hypothesis. For example, H^3-leucine from ribosomes labeled in the intact cell flows into hemoglobin immediately on incubation in the cell-free system (Fig. 3*A*). This represents the completion of already started chains. On the other hand, after 10 min of incubation with C^{14}-valine in the cell-free system, very little N-terminal C^{14}-valine is found (Table 2). This lag coincides with the time required for P^{32}-labeled ribosomes to attach to polyribosomes and begin detaching (Fig. 2). This also corresponds to the lag in polyribosome breakdown shown earlier,[12] that is, between 5–10 min of incubation, ribosome attachment balances ribosome detachment. Secondly, the inhibition by poly A which prevents attachment of single ribosomes does not inhibit the flow of H^3-leucine from ribosomes labeled in the intact cell into hemoglobin (completion of chains). It does not inhibit the initial rate of incorporation either. However, after 60 min of incubation with poly A, hemoglobin synthesis is usually inhibited by 35–40 per cent. The N-terminal value of 3.5 per cent found for hemoglobin synthesized in the cell-free system indicates that 40 per cent of the radioactivity should be in newly synthesized polypeptide chains. This evidence for inhibition of *de novo* synthesis by poly A is confirmed by the marked inhibition of C^{14}-valine incorporation into N-terminal position in the presence of poly A. In contrast, completion and release of

already started polypeptide chains and also polyribosome breakdown[12] are *not* inhibited by poly A, demonstrating the linkage of ribosome detachment and polypeptide chain completion.

The formation of polyribosomes with synthetic or viral polynucleotides has been shown.[5, 6, 9, 10] In these cases, polyribosome formation did not require energy. Some polyribosome formation in the absence of energy, plus an energy-dependent increase, was reported by Goodman and Rich.[13] In our studies, no ribosome attachment to polyribosomes was found in the absence of energy. This may be due to the low level of $MgCl_2$ (10^{-4} *M*) present in the sucrose gradient used in these studies. Since polyribosomes from reticulocytes were quite stable under these conditions, the low concentration of $MgCl_2$ was chosen to avoid the possibility of nonspecific formation of polyribosomes. Specific polyribosome formation should result in attachment of ribosomes at a specific point on the messenger RNA such that polypeptide formation starts at the N-terminal amino acid of the chain. It is difficult to see how such discrimination could be obtained in a nonenzymatic reaction.

It is clear from these studies and those of Goodman and Rich[13] that the attachment of ribosomes fails first in the cell-free system and limits further protein synthesis. The finding that derived ribosomes (80S ribosomes prepared by incubation of polyribosomes) were active in attaching to polyribosomes suggests that failure of attachment is not due to damage of the 80S ribosome, but due to a lesion in the polyribosome structure. Possibly the attachment point of the messenger RNA is the most easily damaged, or some enzymatic reaction as yet unknown is involved in attachment.

Both the amount of inhibition of hemoglobin synthesis by poly A and the amount of N-terminal C^{14}-valine indicate that 40 per cent of the incorporated radioactivity is in newly synthesized chains. The *number* of newly synthesized chains (*de novo* synthesis) is therefore 25 per cent, since each chain synthesized completely in the cell free system contains twice as much radioactivity as the old chains, e.g., the average length of the unlabeled incomplete chain already present in the ribosome is one half.[16] The inhibition of incorporation into *ribosomes* by poly A is about 25 per cent and agrees with this. However, the P^{32} attachment data is too low. About one P^{32} ribosome should attach for each three already in the polyribosome, and the data show only 1 in 10. We feel the data do not reflect the true attachment. The P^{32}-labeled ribosomes are usually poorer for amino acid incorporation than the usual 1X ribosomes. Since they are diluted five fold with unlabeled 80S ribosomes and the calculations assume equal activity, it is likely that actually more ribosomes are attaching in these experiments than the data indicate. In addition, at the time point used for these calculations (5 min), P^{32}-labeled ribosomes may have attached and already left the ribosome, although this number cannot be large. Further work is needed to resolve this point.

Summary.—The attachment of P^{32}-labeled 80S ribosomes to polyribosomes required energy. Studies of the kinetics and amount of *de novo* polypeptide chain synthesis indicated that initiation of the chain required attachment of 80S ribosomes to polyribosomes. This was confirmed by the inhibition of ribosome attachment and new chain synthesis by poly A. Completion of already started chains was correlated with ribosome detachment from the polyribosome.

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THE STRUCTURE OF THE PHENYLALANINE-HYDROXYLATION COFACTOR

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In 1958, we described the purification from rat liver extracts of a cofactor required for the enzymatic conversion of phenylalanine to tyrosine.[1] The results of both chemical and enzymatic studies suggested that the active compound was an unconjugated pteridine, i.e., without a *p*-aminobenzoyl-glutamic acid side chain on carbon 6 of the pteridine ring.[2, 3] This suggestion was supported, for example, by the finding that tetrahydrofolate was only about one fourth as active as the unconjugated pteridine, 2-amino-4-hydroxy-6-methyl tetrahydropteridine.[3]

Recently, it was found that extracts of the *sepia* mutant of *Drosophila melanogaster* had high cofactor activity.[4] An active compound was isolated from these extracts and identified as sepia pteridine[4] (Fig. 1*A*). This was the first naturally occurring unconjugated pteridine which exhibited cofactor activity. It was also reported that biopterin (Fig. 1*B*), another pteridine found in the insect, showed high cofactor activity but only after chemical reduction.[4] These results indicated that the rat liver cofactor was closely related structurally to sepia pteridine and biopterin.

In this report, the results of structural studies on the cofactor purified from rat liver extracts will be presented. The evidence proves that the cofactor is a dihydrobiopterin.

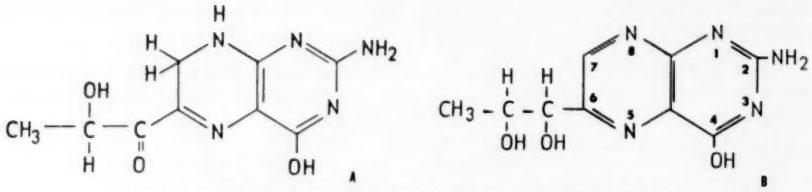

FIG. 1.—Structure of sepia pteridine[5] (*A*), and biopterin (*B*).

Materials and Methods.—Purification of the cofactor: The cofactor was purified by a modification of the published procedure,[1, 3] and assayed as already described.[1] All steps were carried out at 4° in a darkened room. Glass-distilled water was used throughout. The boiled extracts were kept frozen until they were used. The extracts (4 liters prepared from 2,700 gm of rat liver) were thawed and brought to pH 4.0 with 1 *N* H_2SO_4, and the precipitate was removed by centrifugation at 1,200 × *g* for 15 min. The cloudy supernatant fluid was poured onto a 6.5 × 17-cm column of Florisil (100/200 mesh) which had previously been washed with 2% acetic acid until the effluent was pH 4.0. After all of the acidified extract had been added, the column was washed with 1 liter of 2% acetic acid followed by 1 liter of water. The cofactor was eluted with 20% acetone. The flow rate during the elution was adjusted to 1 ml/min and 15–20 ml fractions were collected. The individual fractions were concentrated under reduced pressure, taken up in water, and assayed for cofactor activity. The active fractions were combined and carried through the Dowex 50 (column size = 3.4 × 20 cm), phosphotungstic acid, and silica-gel (column size = 2.6 × 33 cm) steps as previously described.[1, 3]

As a final purification step, the combined eluates from the silica-gel column[6] were concentrated to a small volume (0.4–0.5 ml) and streaked on Whatman 3MM filter paper with a stream of nitrogen directed on the paper. The paper was developed overnight at 4° in *n*-propanol:1% ammonia (2:1). The cylinder was flushed with nitrogen before the paper was introduced. After development, the paper was dried in an atmosphere of nitrogen, and the cofactor eluted from the paper with water. The Rf of the cofactor in this solvent system was about 0.24.

Biopterin (0.5 μmole) was reduced by treatment with Zn dust in 0.25 ml 1 *N* HCl. The mixture was heated for a few minutes at 55°, cooled, and brought to pH 6 with 1 *N* phosphate buffer, pH 6.8. The precipitate of unreacted Zn and Zn phosphate was removed by centrifugation. The supernatant solution was adjusted to pH 2 with HCl and the acidified solution stored at −20°.

All analytical studies on the cofactor were carried out on eluates from the paper chromatogram, using an eluate from an adjacent strip of paper as a blank.

Biopterin was a gift from Dr. H. Rembold of the M. Planck Institute for Biochemistry, Munich, Germany. A small sample of synthetic biopterin was kindly supplied by Dr. E. L. Patterson, American Cyanamid Company. 2-Amino-4-hydroxy-pteridine-6 carboxylic acid and 2-amino-4-hydroxy-pteridine-6 aldehyde were gifts from Dr. T. Shiota, National Institutes of Health. Crystalline yeast alcohol dehydrogenase was purchased from the Worthington Corporation, and Florisil was obtained from the Floridin Company, Hancock, West Virginia.

Results and Discussion.—Properties of the cofactor before and after heating: The absorption spectrum of the purified cofactor is shown in Figure 2, along with that of chemically reduced biopterin. The spectra of the two compounds are approximately the same.[7]

When solutions of the cofactor were heated in the dark at 100° in 0.05 *N* HCl, the spectrum gradually changed until, after 20 min, the spectrum (Fig. 3) resembled that reported for biopterin.[8] This procedure, which inactivated the cofactor, provided a method for estimating the molar concentration of cofactor[9] based on the assumption that the extinction coefficient for the heated compound was the same as that reported for biopterin. (Molar extinction coefficient at 360 mμ in 0.1 *N* NaOH = 7.4×10^3 calculated from the data of Patterson *et al.*[8])

Chromatographic analysis of the heated cofactor showed a major blue-fluorescent

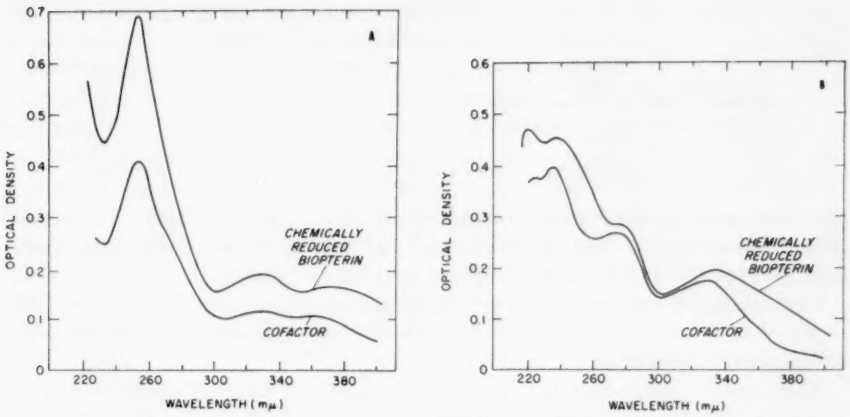

FIG. 2.—Ultraviolet absorption spectrum of the purified rat liver cofactor and of chemically reduced biopterin in 0.1 *N* HCl (*A*), and in 0.1 *N* NaOH (*B*).

spot and one or two minor fluorescent spots. A comparison of the Rf values for the blue-fluorescent spot and for authentic biopterin in several different solvents is shown in Table 1. From these results it is apparent that the cofactor, when heated under these conditions, forms biopterin as the major product.

Permanganate oxidation: It is known that 6-substituted pteridines such as biopterin[10] and sepia pteridine[11] are oxidized by alkaline permanganate to 2-amino-4-hydroxy-pteridine-6-carboxylic acid. The oxidation of the cofactor was carried out in 0.1 *N* NaOH as described for biopterin.[10] After the excess permanganate had been destroyed by ethanol, the mixture was centrifuged. The supernatant solution was neutralized, concentrated, and applied to Whatmann 3MM paper. The chromatogram was developed in *n*-propanol:1 per cent NH_3, 2:1. A single blue-fluorescent spot, Rf = 0.14, could be seen under ultraviolet illumination.

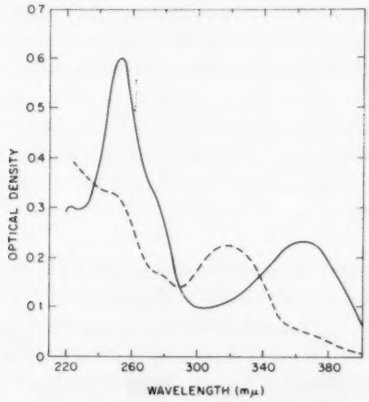

FIG. 3.—Ultraviolet absorption spectrum of the rat liver cofactor after heating at 100° for 20 min in 0.05 *N* HCl; - - -, 0.1 *N* HCl; ——, 0.1 *N* NaOH.

TABLE 1

SUMMARY OF RF VALUES FOR HEATED COFACTOR

Solvent system	Heated cofactor	Biopterin
n-Propanol-1% ammonia (2:1)	0.41	0.43
n-Butanol-acetic acid-water (20:3:7)	0.32	0.33
n-Butanol-acetic acid-water (4:1:1)	0.28	0.28
3% Ammonium chloride	0.65	0.65

Whatman 3MM paper was used. Chromatograms were developed by the ascending technique, at room temperature in the dark.

This fluorescent compound cochromatographed with authentic 2-amino-4-hydroxy-pteridine-6-carboxylic acid in this solvent, as well as in *n*-butanol-water-acetic acid, (5:1:4), Rf = 0.22, and in 3 per cent ammonium chloride, Rf = 0.47.

The absorption spectrum of the permanganate-oxidation product was determined in 0.1 *N* HCl and 0.1 *N* NaOH. The spectra were the same as those reported for the pteridine-6-carboxylic acid.[12]

These results prove that the cofactor is a 2-amino-4-hydroxy-pteridine bearing an oxidizable substituent at the 6 position.

Periodate oxidation: Oxidation of the cofactor by periodate was studied under conditions where biopterin is degraded to 2-amino-4-hydroxy-pteridine-6-aldehyde[10, 13] and acetaldehyde.[10] The periodate reaction was carried out at pH 2.0 and followed spectrophotometrically[14] at 230 mμ. A control eluate from the paper chromatogram served as a blank. The end point of the reaction was taken as the time when the rate of decrease of optical density in the periodate-cofactor cuvette equaled that of the periodate-paper blank cuvette. The reaction was usually over after 2 hr. It was found that the cofactor before boiling consumed 2.10 μmoles of periodate/μmole, while after boiling this figure was 1.09 μmoles/μmole. This result indicated that one half of the periodate consumption observed with the cofactor before boiling was due to oxidation of the reduced pteridine ring, while the other half was due to cleavage of the side-chain.

The absorption spectrum of the heated cofactor after treatment with periodate was determined in 0.1 *N* NaOH. It was similar to that reported for 2-amino-4-hydroxy-pteridine-6-aldehyde[10, 13] (peaks at 255, 275, and 365 mμ).

The periodate-reaction mixture was concentrated and applied to Whatman 3MM paper. The chromatogram was developed overnight in *n*-butanol-acetic acid-water (20:3:7). The single blue-fluorescent spot (Rf = 0.28) was somewhat elongated, probably due to the presence of salts. Carbonyl groups were determined on the fluorescent compound (after elution from paper) by the 2,4-dinitro-phenyl-hydrazine method.[15] Authentic 2-amino-4-hydroxy-pteridine-6-aldehyde served as the standard. The compound had 0.97 μmoles of carbonyl group per μmole of cofactor. Before treatment of the cofactor with periodate, no carbonyl groups could be detected.

If the cofactor is a biopterin derivative, the other expected product of the periodate reaction should be acetaldehyde. The periodate reaction was carried out under the same conditions as described for the assay of threonine.[16] The liberated acetaldehyde was determined enzymatically by measurement of the amount of DPNH oxidized in the presence of alcohol dehydrogenase.[16] The cofactor yielded 0.79 μmoles of acetaldehyde/μmole. Authentic biopterin, taken through the same procedure, yielded 0.80 μmole acetaldehyde/μmole.

The acetaldehyde formed during the periodate reaction was also determined by

the Barker-Summerson[17] procedure. Before color development, the excess periodate was destroyed by the addition of 0.01 ml of 1 *M* thiopropionic acid. The amount of acetaldehyde found was 0.71 μmoles/μmole cofactor.

Acetaldehyde was identified as one of the reaction products by thin-layer chromatography on silicic acid. An aliquot of the periodate reaction mixture after treatment with thiopropionic acid was incubated for 10 min at 55° with one-fifth volume of 0.1 per cent dinitrophenylhydrazine in 2 *N* HCl. The dinitrophenylhydrazones were extracted into carbon tetrachloride. The organic solvent was evaporated and the dry residue dissolved in 0.1 ml ethanol. The ethanol solution was applied to the silicic acid-coated plate which was then developed in *n*-heptane saturated with methanol.[18] The major yellow spot had the same Rf (0.20) as the dinitrophenylhydrazone prepared from acetaldehyde.

Iodine titration: The state of reduction of both the cofactor and the chemically reduced biopterin was determined by iodine titration. The results (Table 2) show that both compounds are at the dihydro level.

TABLE 2

IODINE TITRATION OF THE COFACTOR AND OF CHEMICALLY REDUCED BIOPTERIN

	Iodine consumption (μ equivalents/μmole)
Cofactor	1.05
Reduced biopterin	1.01

The titrations were carried out with 0.0005 *N* I_2 solutions in 0.1 *N* HCl with an internal starch indicator.

Biological assay of the cofactor for biopterin: The trypanosomid flagellate, *Crithidia fasciculata*, shows a nutritional requirement which can be satisfied most efficiently by biopterin[13] or very closely related pteridines.[19] Through the generosity of Drs. Dewey and Kidder, Amherst University, and Dr. H. Rembold, Munich, Germany, samples of the purified rat liver cofactor were assayed for biopterin content with this organism. In the two independent determinations, the cofactor was 66 per cent and 185 per cent as active as authentic biopterin (based on the amount of 2-amino-4-hydroxypteridine-6-carboxylic acid obtained from the cofactor on oxidation with permanganate).

The results of the chemical studies prove that the cofactor is a dihydrobiopterin, i.e., dihydro-2-amino-4-hydroxy-6 (1,2-dihydroxypropyl)-pteridine.

The results of the *Crithidia* assay establish the optical configuration of the side chain of the cofactor as the L-erythro isomer.[8] The structure of the cofactor is, therefore, dihydro-2-amino-4-hydroxy-6-[1,2-dihydroxypropyl-(L-*erythro*)]-pteridine.

Enzyme studies: The only uncertainty regarding the structure is the localization of the double bonds in the pteridine ring. The problem is complicated by the many possibilities for tautomerism in this type of compound (see refs. 20 and 21 for a discussion).

The recent demonstration[20] that tyrosine formation in the presence of the cofactor is dependent on dihydrofolic reductase (DHF reductase) indicates that the cofactor is a substrate for this enzyme and therefore exists in the proper dihydro configuration demanded by this enzyme's specificity. There is, however, conflicting evidence regarding this specificity of DHF reductase. It has been reported that 5,8-dihydrofolate[22, 23] is the tautomeric form which serves as the substrate, but there is also evidence in favor of the 7,8-dihydro configuration.[24] In view of this uncertainty, the double bonds in the cofactor can only be tentatively assigned—the cofactor is either 5,8- or 7,8-dihydrobiopterin.

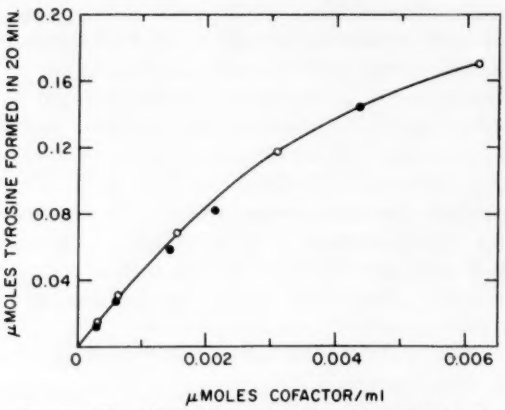

FIG. 4.—The stimulation of tyrosine formation by the rat liver cofactor (○) and by chemically reduced biopterin (●).

A comparison of the activity of the cofactor and of chemically reduced biopterin in stimulating tyrosine formation is shown in Figure 4. No significant difference between the two compounds was detected. The Km for the cofactor, as determined by the Lineweaver-Burk method, is about $6.5 \times 10^{-6} M$.

The pteridine transformations believed to occur during the enzymatic conversion of phenylalanine to tyrosine are shown in Figure 5 where XH_2 represents the dihydropteridine and XH_4 the tetrahydropteridine.

The evidence in favor of a quinonoid-dihydropteridine structure (Fig. 6) for the primary oxidation product of the tetrahydropteridine is based on studies carried out with the model compound, 2-amino-4-hydroxy-6,7-dimethyl tetrahydropteridine.[21] It seems likely that the same type of dihydro compound is formed as the primary product when the cofactor is used.

The scheme explains the pronounced lag period characteristic of the phenylalanine-hydroxylating system.[25] The lag is probably due to the time required for the initial DHF-reductase-catalyzed reduction of the cofactor. Once the reaction has occurred, DHF-reductase should no longer be required since it is unnecessary for the catalytic participation of the cofactor in the hydroxylation reaction.

The most sensitive site of action of the antifolic compound, aminopterin, has been

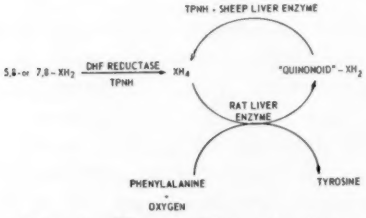

FIG. 5.—The pteridine transformations during the enzymatic conversion of phenylalanine to tyrosine.

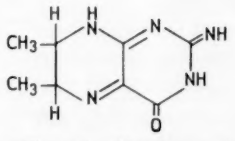

FIG. 6.—Structure of the primary oxidation product of the tetrahydropteridine formed during the enzymatic conversion of phenylalanine to tyrosine.

reported to be DHF-reductase. The enzyme is completely inhibited by concentrations of the drug as low as 5×10^{-7} M.[26]

The reaction sequence outlined in Figure 5 predicts that the sensitivity of the hydroxylation reaction to aminopterin would depend on the time of addition of the drug; at zero time, the system should be inhibited by low concentrations of aminopterin; after the DHF-reductase-catalyzed reaction has taken place, the sensitivity to the drug should be markedly diminished.

The results summarized in Table 3 show that these predictions were fulfilled.

TABLE 3

INHIBITION OF PHENYLALANINE-DEPENDENT OXIDATION OF TPNH BY AMINOPTERIN (4×10^{-7} M)

Time of preincubation before aminopterin (min)	Inhibition (per cent)
0	98
12	10
22	0

The complete reaction mixture contained the following components (in μm): potassium phosphate buffer, pH 6.8, 100; L-phenylalanine, 2.0; TPNH, 0.15; purified cofactor, 0.0015; rat liver enzyme, 0.3 mg protein; sheep liver enzyme, 0.1 mg protein; glucose dehydrogenase, 400 units. Final volume, 1.0 ml. Temperature, 22°. A cuvette in which phenylalanine was omitted served as a control.

In this experiment, the hydroxylation reaction was assayed by measurement of the phenylalanine-dependent oxidation of TPNH.[25] When added at zero time, aminopterin at 4×10^{-7} M inhibited the reaction almost completely; after 22 min, the reaction was completely insensitive to this concentration of the drug. At the later time, significant inhibition could still be observed with 1,000 times more aminopterin. At these high concentrations of the drug, the rat liver enzyme or the sheep liver enzyme are sensitive to the inhibitor.

This result supports the idea that DHF-reductase is not involved in the hydroxylation reaction once the cofactor has been reduced to the tetrahydro level.

Insight into the state of reduction of the cofactor *in vivo* can be obtained by a comparison of the sensitivity of the phenylalanine-hydroxylating system to antifolic compounds *in vivo* and *in vitro*.

Studies of phenylalanine-tolerance tests in man indicate that this procedure reflects the *in vivo* rate of conversion of phenylalanine to tyrosine. Thus, phenylketonuric subjects, lacking a functional phenylalanine-hydroxylating system, show a marked change in phenylalanine tolerance, while heterozygotes (i.e., parents of phenylketonuric children), presumably having reduced levels of the enzyme, show less of a change in phenylalanine tolerance.[27, 28]

Results obtained with nonphenylketonuric subjects after amethopterin administration were similar to those of heterozygotes,[29] an indication that the hydroxylase activity was only partially inhibited by the drug. This result suggests that, *in vivo*, the cofactor is at least partially, and perhaps predominantly, in a form which does not require DHF-reductase for its activity (i.e., the cofactor is either in the tetrahydro or the quinonoid-dihydro form).

This suggestion is also supported by the finding that both the dependence on DHF-reductase and the sensitivity of the hydroxylation reaction to aminopterin vary with the purity of the cofactor. Thus, the *in vitro* hydroxylation reaction in the presence of the crude cofactor shows little stimulation by DHF-reductase, while in the presence of the purified cofactor, the system exhibits an almost absolute requirement

for this enzyme.[20] Moreover, with the purified cofactor the hydroxylation reaction is inhibited by 4×10^{-7} *M* aminopterin (see Table 3), while with the crude cofactor, 1,000 times more aminopterin is required for significant inhibition.[3] These results indicate that the cofactor in crude extracts exists in either the tetrahydro or the quinonoid-dihydro form. During purification, it is gradually converted to the form which requires DHF-reductase for activation, i.e., the 5,8- or 7,8-dihydro form.

With the elucidation of the structure of the phenylalanine hydroxylation cofactor as dihydrobiopterin, the first metabolic role for an unconjugated pteridine has been established. There are already indications that this class of compound may play a similar role in other oxidative reactions.[30]

Summary.—The structure of the phenylalanine hydroxylation cofactor has been established as dihydrobiopterin. The role of the cofactor in the hydroxylation reaction has been discussed.

The author wishes to thank Mr. Richard Funk for skillful technical assistance during the purification work.

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[6] Some cofactor activity, usually 20–25% of the total, is found in a peak eluted from the silica gel column just behind the solvent front. This material has not been combined with the main peak. It is not known if the apparent separation of the activity into two peaks is an artifact of the column chromatography or if the early peak represents a different compound with cofactor activity.

[7] The spectrum of the cofactor resembles, to some extent, that reported for a tetrahydrobiopterin derivative (perhaps a riboside) isolated from the eyes and skin of amphibia, as well as from the eyes of insects [see I. Ziegler, *Z. Naturforsch.*, **15b,** 460 (1960)]. It is possible that the spectrum reported by Ziegler is that of a mixture of tetrahydro and dihydrobiopterin derivatives.

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CONSERVATION OF SPECIFICITY BETWEEN AMINO ACID ACCEPTOR RNA AND AMINO ACYL-sRNA SYNTHETASE

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Several amino acids can be attached to the soluble RNA (sRNA) of one organism by amino acyl-sRNA synthetases of other organisms.[1–4] The nature of the cross reactions can be studied further by chromatographically identifying the amino acyl-sRNA formed under such conditions. Some results obtained by methylated albumin column fractionation have been reported.[5] Further studies with column fractionation of amino acyl-sRNA, formed by the interspecific combination of sRNA and the synthetase, have resulted in one of the following situations: (*a*) no cross reaction was observed; (*b*) the same profile was secured as that of normal amino acyl-sRNA; (*c*) only some component or components of normal amino acid acceptor RNA were charged; (*d*) an entirely different profile was noted. The first three are commonly observed. When cross reactions are observed, normal components of sRNA for the particular amino acid are charged with the amino acid. The last situation occurs in only one case so far examined where yeast leucyl-sRNA is formed by an *E. coli* synthetase; the lcueyl-sRNA formed has an entirely different profile from those of normal yeast and *E. coli* leucyl-sRNA's. However, the significance of this exception is not clear, since the leucyl-sRNA obtained constitutes only one per cent of the normal yeast leucyl-sRNA. These results indicate that the specificity between sRNA and the activating enzyme for each amino acid is strikingly conserved among different organisms. This conservative feature is even more remarkable when the adaptor hypothesis for the role of sRNA in protein synthesis is considered.

Materials and Methods.—Bacteria: The following strains were used: *Escherichia coli* B, *Pseudomonas aeruginosa* (American Type Culture Collection #10197), *Bacillus subtilis* (W23), *Micrococcus lysodeikticus* (ATCC #4698), *Aerobacter aerogenes* (ATCC #9624), *Salmonella typhimurium* (LT-2). Yeast: a strain of baker's yeast.

Preparation of sRNA: The sRNA was prepared by the phenol procedure described by von Ehrenstein and Lipmann.[6] To remove any attached amino acids, the sRNA preparation was incubated in 0.5 *M* Tris HCl, pH 8.8, for 45 min at 37°C. This suspension was then brought to 1 *M* of NaCl concentration, and sRNA precipitated by the addition of 2 vol ethanol, dissolved in H_2O, dialyzed against cold distilled water overnight, and lyophilized.

Preparation of enzyme extract: Essentially the method of Takanami and Okamoto[7] and Zubay[8] was used. Bacteria were grown at 37°C with constant shaking in enriched broth and yeast in a medium containing glucose (4%), peptone (0.5%), yeast extract (0.25%), ammonium sulfate (0.2%), KH_2PO_4 (0.1%), $MgSO_4$ (0.025%), and $CaCl_2$ (0.025%), pH adjusted to 4.8–5.0 (HCl)

The cells were harvested in the logarithmic phase (A_{650}:0.3–0.5) and ground with 3 times their wet weight of alumina (levigated alumina from Norton Abrasives, Worcester, Mass.). To the crude extract from a one-liter culture, 3 ml of Tris-magnesium buffer (0.01 *M* Tris-HCl buffer, pH 7.3 plus 0.01 *M* $MgCl_2$) were added, and the mixture was centrifuged at 105,000 × *g* for 3 hr at 0°C. The upper 2/3 of the supernatant was dialyzed against 500 ml Tris-magnesium buffer plus 0.006 *M* mercaptoethanol at 4°C for 3 hr, changing the outside buffer every half hour.

Preparation of enzyme fraction free from RNA: 105,000 × *g* supernatant was applied to a 1.2 cm × 5 cm column of DEAE cellulose equilibrated with phosphate buffer (0.02 *M*, pH 7.7) containing 0.006 *M* mercaptoethanol. The charged column was rinsed with 50 ml of the phosphate buffer, and the enzyme eluted with 0.02 *M* potassium phosphate buffer containing 0.35 *M* NaCl (pH 7.7). Fractions were collected in 2 ml portions, and tubes with high absorbancies were combined and used for esterification of amino acids to sRNA.

Preparation of amino acyl-sRNA: C^{14}-labeled amino acyl-sRNA was prepared basically according to Berg *et al.*[3] The reaction mixture contained the following compounds totaling 0.5 ml: 50 μmoles of Tris buffer (pH 7.3); 0.5 μmole of ATP, 5 μmoles of $MgCl_2$; 0–1 mg of sRNA; 2 μmoles of reduced glutathione; 5 μmoles of KCl, 0.01–0.02 ml of enzyme extract, an appropriate amount of C^{14}-amino acid plus 19 remaining nonradioactive amino acids (1 μmole of each); and, unless otherwise stated, the reaction mixture was incubated at 37°C for 25 min. Amino acyl-sRNA was isolated by the phenol procedure of Gierer and Schramm.[9] Radioactive amino acids used were: from Calbiochem, Los Angeles, Calif., L-methionine 4.5 μc/μmole (specific activity); from New England Nuclear Corp., Boston, Mass., L-leucine C^{14} 143.4 μc/μmole, L-lysine C^{14} 144 μc/μmole, L-phenylalanine C^{14} 369 μc/μmole, L-proline C^{14} 115 μc/μmole, L-methionine H^3 14.1 μc/μmole, DL-leucine H^3 5400 μc/μmole, DL-phenylalanine H^3 30 μc/μmole, DL-proline H^3 5000 μc/μmole.

Incorporation experiments: Essentially the method of Nirenberg and Matthaei[10] was followed for the preparation of *E. coli* extracts (preincubated, DNAase-treated, S-30 fractions) and for reaction mixtures used for determining C^{14}-amino acid incorporation into protein. The reaction mixture (0.5 ml) contained the following components: 50 μmoles Tris pH 7.8; 5 μmoles magnesium acetate; 25 μmoles KCl; 3 μmoles mercaptoethanol; 25 μmoles *M* PEP; 10 μg of PEP-kinase (Calbiochem); 0.15 μmoles GTP; 10 μg of polynucleotide; 0.05 mg of C^{12}-amino acyl-sRNA omitting leucine; 0.02 ml of the incubated S-30 fraction, and C^{14}-leucyl-sRNA. The poly UC (base ratio 2.1:1) and UG (2.8:1) were kindly donated by Dr. M. W. Nirenberg, and poly U by Dr. J. Fresco.

Methylated albumin column: The preparation of this column was simplified by pouring 30 ml of MAK (mixture of kieselguhr and methylated albumin in phosphate buffer) directly into the column (31 mm i.d.) as the first layer, and a suspension of 1 gm kieselguhr in 0.2 *M* saline buffer on top as a protective layer. The MAK was prepared by suspending 6 gm of kieselguhr in 30 ml of 0.05 *M* sodium phosphate buffer, pH 6.7, followed by boiling and cooling the suspension. 1.5 ml of 1% methylated albumin solution in H_2O was stirred in slowly.[11]

Results.—Our approach to the study of the nature of interspecific cross reaction between sRNA and the amino acyl-sRNA synthetase is qualitative as well as quantitative; namely, the nature of the cross reaction is examined from elution patterns of amino acyl-sRNA on a methylated albumin column. Soluble RNA's were isolated by the phenol method, and extracts containing amino acyl-sRNA synthetases free from RNA were prepared by chromatography on a DEAE-cellulose column. Extracts were checked for enzyme activity using homologous sRNA. Contamination of homologous sRNA in the extract was proved negligible: P^{32}-labeled sRNA mixed with crude extract was totally retained on the DEAE cellulose column after elution with an 0.35 *M* saline buffer solution. Contamination was also examined from time to time by incubating the fractionated enzyme extract with the radioactive amino acid in question, adding homologous nonradioactive sRNA as a carrier, and immediately isolating the sRNA by the phenol method. Fractionation of such RNA gave no radioactive peak.

In Table 1 the esterifying of a radioactive amino acid to sRNA by the heterologous enzyme is expressed in percentage against that by the homologous enzyme.

Phenylalanine: As shown in Figure 1, the perfect matching of the two yeast phenylalanyl-sRNA preparations, one using a homologous (yeast) enzyme and the other a heterologous (*E. coli*, or *Ps. aeruginosa*) enzyme, indicates that amino acyl-sRNA synthetases from the three sources have the same specificity. A similar effect is observed with *E. coli* sRNA and the enzymes from *Ae. aerogenes*, *B. subtilis*, and *M. lysodeikticus*. In the *E. coli*-yeast combination, yeast amino acyl-sRNA synthetase fails to esterify phenylalanine to *E. coli* sRNA, although the *E. coli* enzyme is able to attach phenylalanine onto yeast sRNA. The failure of *E. coli* sRNA to accept phenylalanine by yeast enzyme could be attributed to a special RNAase present in the yeast enzyme extract, which partially degrades *E. coli* sRNA, or, more specifically, the acceptor end of the sRNA. This possibility was examined by checking the phenylalanine acceptor activity using *E. coli* amino acyl-sRNA synthetase. No difference was observed, either qualitatively or quantitatively, between *E. coli* sRNA treated or untreated with yeast enzyme extract (Fig. 2), indicating that the action of RNAase, which may exist in yeast extract, is unlikely.

TABLE 1

EXTENT OF AMINO ACID ATTACHMENT TO sRNA BY HETEROLOGOUS AMINO ACYL-sRNA SYNTHETASES

sRNA	Synthetase	Methionine	Phenyl-alanine	Leucine	Lysine	Proline
E. coli	*E. coli*	100%	100%	100%	100%	100%
	Yeast	37	2	74	59	0.9*
	S. typhimurium (LT-2)		91*	95*		
	B. subtilis (W23)		99*			
	Ae. aerogenes		99*			
	M. lysodeikticus		58*			
Yeast	Yeast	100%	100%	100%	100%	100%
	E. coli	60	12	0.8	70	3*
S. typhimurium (LT-2)	*S. typhimurium* (LT-2)		100%	100%		
	E. coli		81*	104*		

Relative amino acid acceptor activity of *E. coli*, yeast, and *S. typhimurium* sRNA's when assayed with enzyme extracts from various sources. Assay conditions are as described under *Materials and Methods*. The values given are the average of several experiments, normalized to the value obtained for each sRNA with homologous enzyme. The figures with asterisks are based on one set of experiments.

Proline: Amino acyl-sRNA synthetase from *E. coli* could not utilize yeast sRNA, and vice versa (Fig. 3).

Leucine: Previously we reported that the leucyl-sRNA of yeast formed by the enzyme of *E. coli*, although small in amount, is different in profile from the normal yeast leucyl-sRNA,[12] and that leucyl-sRNA of *E. coli* formed by the yeast enzyme has a profile covering the front part of the normal *E. coli* leucyl-sRNA.[5] Figures 4*A* and 5*A* show that the amount of leucyl yeast-sRNA formed by the *E. coli* enzyme is about one per cent of that formed by yeast enzyme. As shown in Figure 5, the amount of leucyl-sRNA thus formed is proportional to the amount of yeast sRNA in the reaction mixture. The reciprocal combination, *E. coli* sRNA and yeast enzyme, formed leucyl-sRNA in about two thirds of the normal *E. coli* quantity. The elution profile from the methylated albumin column is shown in Figures 6*A* and 6*B*. The cause of the "strange" leucyl-sRNA profile observed in yeast-

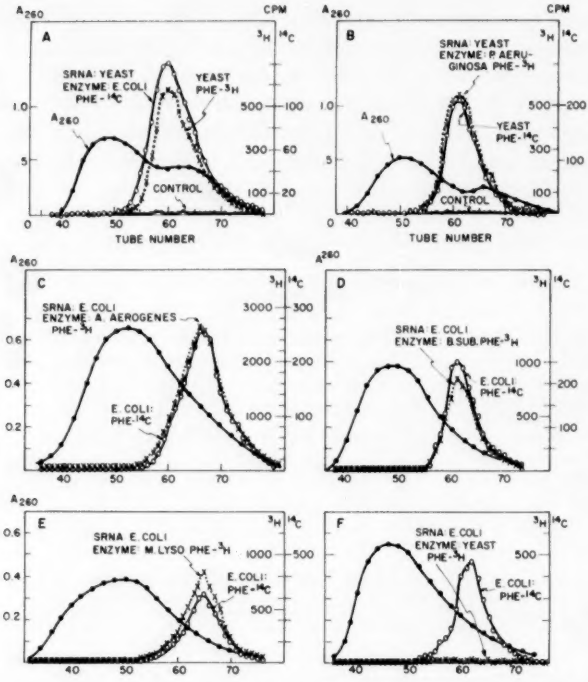

FIG. 1.—Interspecific exchange of sRNA and the amino acyl-sRNA synthetase for the formation of phenylalanyl-sRNA. Comparison of the chromatographic profiles of normal yeast C^{14}-phenylalanyl-sRNA and yeast H^3-phenylalanyl-sRNA obtained, using *E. coli* enzyme extract (*A*), and *Ps. aeruginosa* (*B*), normal *E. coli* C^{14}-phenylalanyl-sRNA with *E. coli* H^3-phenylalanyl-sRNA formed with enzyme extract from *Ae. aerogenes* (*C*), *B. subtilis* (*D*), *M. lysodeikticus* (*E*), and yeast (*F*). For comparison, normal *E. coli* or yeast phenylalanyl-sRNA was mixed with the product of heterologous enzymes and chromatographed on a simplified methylated albumin column. The differential counting of C^{14} and H^3 was done in a Packard Tri-Carb liquid scintillation counter. For control, no sRNA was added during the incubation period, but after chilling and addition of cold phenol the same amount of sRNA was added as in the heterologous combination. The sRNA was isolated and chromatographed as in the text.

sRNA and the *E. coli* enzyme combination may be attributed to one of the following possibilities: (1) radioactive leucine preparations being contaminated by another amino acid; (2) yeast sRNA being modified by some enzymes present in the *E. coli* enzyme extract; (3) the "strange" peak being a minor component of normal yeast leucyl-sRNA; (4) leucine being esterified to a yeast sRNA which normally does not bind leucine. The first possibility is not likely, because removal of 19 other nonradioactive amino acids from the reaction mixture did not change the

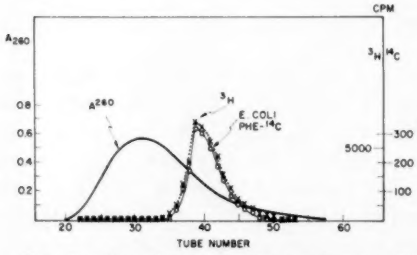

FIG. 2.—*E. coli* sRNA after treatment with yeast enzyme extract as described in *Methods*, tested for phenylalanine (H^3) acceptor activity using homologous (*E. coli*) enzyme. *E. coli* C^{14}-phenylalanyl-sRNA added for comparison.

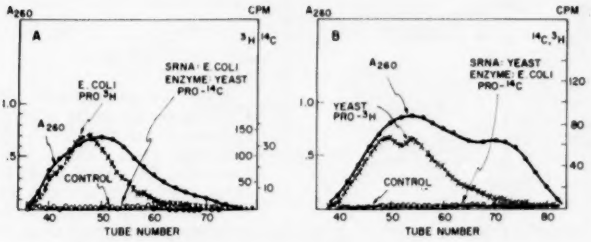

FIG. 3.—Comparison of chromatographic profiles of (*A*) *E. coli* H^3-prolyl-sRNA and *E. coli* sRNA treated with yeast enzyme extract for the attachment of C^{14}-proline; (*B*) yeast H^3-prolyl-sRNA and yeast sRNA treated with *E. coli* amino acyl-sRNA synthetase for the binding of C^{14}-proline.

result (100 times in excess of radioactive leucine), while the addition of nonradioactive leucine eliminated the peak. As to the second possibility, Nishimura and Novelli[13] reported that the treatment of *E. coli* leucyl-sRNA with *B. subtilis* RNAase causes a shift in the methylated albumin-kieselguhr chromatographic profile. To test this possibility, yeast sRNA charged with C^{14}-leucine by the *E. coli* enzyme was discharged by pH 8.8 incubation and recharged with C^{14}-leucine, using the homologous enzyme. The elution profile of the resulting sRNA is shown with the normal yeast H^3-leucyl-sRNA (Fig. 7). The perfect fitting of the two leucyl-sRNA's disproved the second possibility. In this case, since the reaction mixture for treating yeast sRNA with the *E. coli* enzyme contained methionine and ATP, the possible effect of methylation on the profile should have been noted in the final elution pattern. The absence of modification of the profile tended to exclude also the methylation of yeast sRNA by the *E. coli* enzyme extract. The third possibility was examined by refractionating the "strange" peak to see whether a component of the normal leucyl-sRNA corresponded to the "strange" peak. Owing to the small amount of the "strange" component, the result was not conclusive. The last possibility also cannot be excluded. In spite of various experiments attempting to identify the "strange" peak as a normal component among the yeast amino acid acceptor RNA, the results have not been clear. The transfer of leucine from the "strange" peak to poly UC and poly UG are shown in Figure 8*A*.

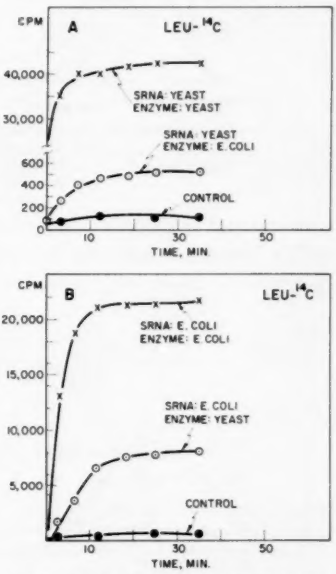

FIG. 4.—Kinetics of leucyl-sRNA formation. The conditions used were those described for the usual assay of amino acyl-sRNA formation. For control, no sRNA was added during the incubation period, but after chilling and addition of cold 10% TCA, 0.1 mg of sRNA was added.

Methionine: The interspecific exchange of sRNA and enzyme between *E. coli* and yeast shows that only a part of the methionine acceptor RNA of each organism can be charged with heterologous enzyme (Fig. 9*A*). The result on the methionyl-sRNA formed by *E. coli* sRNA and yeast enzyme confirms the original finding of Berg *et al.*[3] and shows that a similar situation exists for the reciprocal

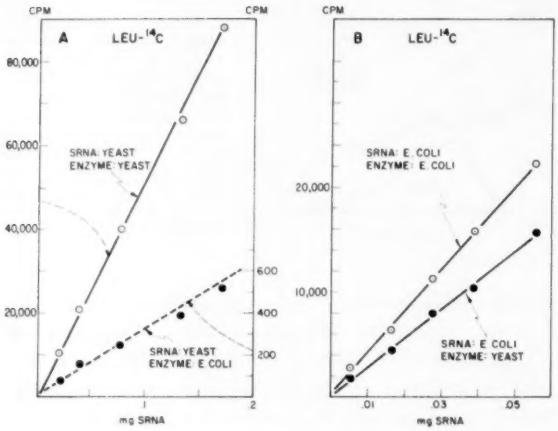

FIG. 5.—Attachment of C^{14}-leucine to yeast sRNA (*A*) and *E. coli* sRNA (*B*) by enzymes from either organism. In reaction mixtures, the same amount of C^{14}-leucine was added to the homologous and heterologous combination. Incubation was made at 37°C for 30 min, and the reaction was stopped by adding 10% TCA (final concentration).

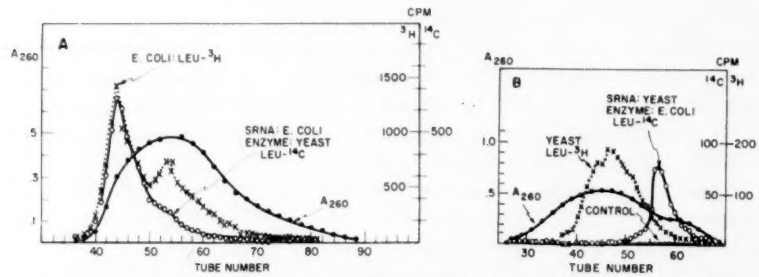

FIG. 6.—Comparison of the chromatographic profiles of (*A*) *E. coli* H^3-leucyl-sRNA and *E. coli* C^{14}-leucyl-sRNA obtained by using yeast amino acyl-sRNA synthetase, and (*B*) yeast H^3-leucyl-sRNA and yeast C^{14}-leucyl-sRNA formed by *E. coli* enzyme extract.

combination (Fig. 9*B*). In our previous report,[12] we mentioned that the methionyl-sRNA formation between *E. coli* and yeast showed a "strange" peak as in the case of leucine. However, the result was proved wrong since the enzyme extract had been contaminated with homologous sRNA.

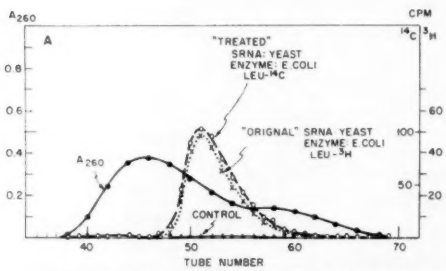

FIG. 7.—Effect of *E. coli* extract on the profile of the "strange" yeast leucyl-sRNA formed by *E. coli* extract. "Treated": yeast sRNA, after treatment with *E. coli* enzyme extract and H^3-leucine, was discharged and recharged with C^{14}-leucine esterified using *E. coli* enzyme. "Original": yeast sRNA charged with H^3-leucine by *E. coli* enzyme.

Lysine: Between *E. coli* and yeast, the profile of the lysyl-sRNA formed by heterologous enzymes showed exactly the same profiles as those of the normal lysyl-sRNA (Fig. 10).

Discussion.—The chromatographic analysis of the amino acyl-sRNA produced by interspecific cross reactions between sRNA and the amino acyl-sRNA synthetase indicated that all or some of the normal components of amino acyl-

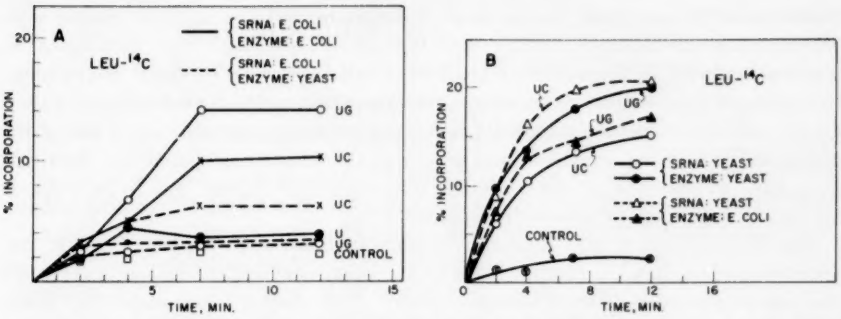

FIG. 8.—(*A*) Polymer-dependent incorporation of *E. coli* C^{14}-leucyl-sRNA obtained by using homologous and yeast enzymes. See text for conditions. Each point represents: —○—○—, —x—x—, —●—●—, 0.020 mg of sRNA with 7,728 cpm; ---○---○---, ---x---x---, ---●---●---, 0.035 mg sRNA containing 6,541 cpm. Control contained all components, except polymer. (*B*) Polymer-dependent incorporation of yeast C^{14}-leucyl-sRNA obtained by using homologous and *E. coli* enzymes. Each point represents: —○—○—, —●—●— 0.014 mg of sRNA, 7,224 cpm; ---△---△---, ---▲---▲--- 0.98 of sRNA, 3,274 cpm.

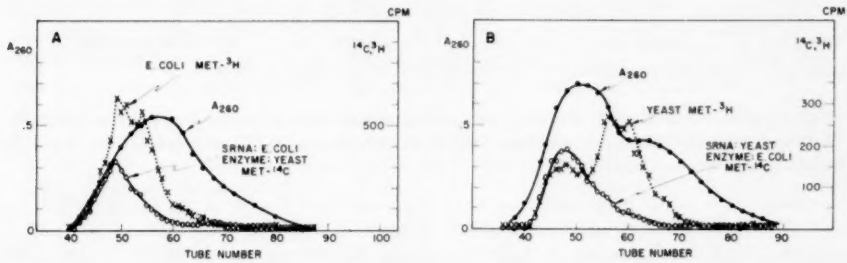

FIG. 9.—Comparison of the chromatographic profiles of (*A*) *E. coli* H^3-methionyl-sRNA and *E. coli* C^{14}-methionyl-sRNA obtained by using yeast enzyme extract; (*B*) yeast H^3-methionyl-sRNA and yeast C^{14}-methionyl-sRNA formed by *E. coli* enzyme.

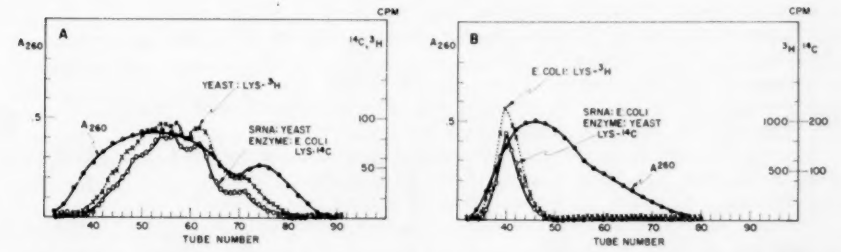

FIG. 10.—Comparison of the chromatographic profiles of (*A*) normal yeast H^3-lysyl-sRNA and yeast C^{14}-lysyl-sRNA obtained using *E. coli* enzyme; (*B*) normal *E. coli* H^3-lysyl-sRNA and *E. coli* C^{14}-lysyl-sRNA formed by yeast enzyme.

sRNA were charged with the particular amino acid. One possible exception has been found in the formation of yeast leucyl-sRNA by the *E. coli* enzyme. However, owing to the relatively small amount, the nature and significance of this exception is not clear.

The conserved specificity is quite surprising; in the adaptor hypothesis the

conservation of specificity in the level of amino acyl-sRNA and the amino acyl-sRNA synthetase is not required, even if the code is universal.[6, 14–16] In view of the unusual behavior on the column of yeast leucyl-sRNA formed by the *E. coli* enzyme, the cross reaction should be examined more extensively before a general conclusion is given. However, the fortuitous fitting between the enzyme-recognizing site of the acceptor RNA of one organism and a part of the activating enzyme of the other organism, irrespective of the amino acid specificity,[12] turned out to be unlikely among the organisms used in the present work.

Bennett, Goldstein, and Lipmann[17] report that although a part of the *E. coli* leucine acceptor RNA is compatible with the yeast enzyme, the *E. coli* enzyme did not charge the yeast leucine acceptor RNA. This does not contradict our result on the same case, since only a small amount of yeast leucyl-sRNA (about 1% of the normal case) is formed by the *E. coli* enzyme.

The first indication of the conserved feature of the specificity was reported by Berg *et al.*[3] who showed that the isolated methionyl-sRNA synthetase from yeast could attach methionine to a part (40%) of the *E. coli* methionine acceptor RNA. Recently, Bennett *et al.*[17] analyzed, in greater detail, yeast leucyl-sRNA formed by *E. coli* enzyme. Their result shows that only a component of the *E. coli* leucine acceptor RNA (peak I of Weisblum *et al.*[18]) accepts leucine by yeast enzyme. Our data for the same combinations are consistent with their results.

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*ON THE NATURE OF NUCLEOLAR RNA**

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It is now generally held that the structural RNA of cytoplasmic ribosomes is of nuclear origin. Several kinds of studies, including autoradiography, have implied that the nucleolus plays a role in ribosomal RNA economy.[1–4] Biochemical investigations have shown that an RNA of base composition similar to that of cytoplasmic ribosomal RNA[5] and of identical molecular sizes, namely, 28 and 18 S, occurs in the nucleolus.[6] In addition, ribosome-like particles have been observed in the nucleolus by electron microscopy[7–11] and have been shown to have sedimentation constants similar to those of cytoplasmic ribosomes and their subunits.[6]

The hybridization of RNA with denatured DNA provides a tool for the identification of DNA complementary in base sequence to any given RNA.[12–16] Application of this technique has enabled us to discover that nucleolar RNA is identical to cytoplasmic ribosomal RNA in base sequence and may therefore be the nuclear precursor of cytoplasmic RNA. We will also show that the DNA regions, or stretches complementary to cytoplasmic ribosomal RNA, are not confined to the nucleolus but are distributed throughout the chromatin of the nucleus.

Materials and Methods.—P^{32} (as orthophosphate) was obtained from the Oak Ridge National Laboratories, Oak Ridge, Tenn. RNase was purchased from Sigma Chemical Co., St. Louis, Mo. T4 DNA was a gift from Dr. Roger Weil of the California Institute of Technology, Pasadena, Calif. Membrane filters type A coarse, Lot no. 2427, were obtained from Schleicher and Schuell, Keene, N. H.

Preparation of labeled RNA: Fifty gm of peas were sterilized with detergent, germinated for 12 hr, rinsed thoroughly with distilled water containing 1 mg penicillin-G per ml, and incubated in a shallow dish 24–36 hr in the presence of 3–5 Cm 95% radiophosphorus (as sodium phosphate) in 10 ml water containing 50 μg penicillin per ml. After incubation, the seedlings were washed, and the P^{32} was chased for 12 hr with 0.001 *M* sodium phosphate, pH 7.0 (10,000-fold excess of P^{31}). The seedlings were then harvested and ribosomes prepared according to the protocol of Ts'o *et al.*[17] The repeated recycling of the ribosomes selects 80 S particles and provides preparations virtually free of sRNA (as determined by sucrose gradient centrifugation). The ribosomal RNA was deproteinized twice according to the procedure of Wallace *et al.*,[18] and once with an equal volume of 88% phenol according to Kirby. The RNA preparations were then fractionated by the sucrose gradient centrifugation described earlier.[19] The 28 and 18 S RNA components (peak fractions) were collected, and each was further purified by a second sucrose gradient centrifugation. The labeled ribosomal RNA subunits were then centrifuged through 3 ml of a CsCl solution, pH 7.0 (1.73 gcm^{-3}) in a Spinco SW 39 at 35,000 rpm for 12 hr in the cold[20] in order to free the RNA of any contaminating DNA. The specific activities of the two RNA components were identical in each preparation and ranged from 5×10^6 to 30×10^6 cpm/mg in different experiments.

Preparation of DNA: Pea nuclei were prepared[21] and purified[6] as described earlier. Nucleoli were obtained by grinding nuclei in sucrose media containing citrate[22] and purified by sedimentation through 2.2 *M* sucrose at 20,000 rpm for 20 min in the SW 25 Spinco head.

DNA was prepared from nuclei and nucleoli by a modification of Marmur's method.[23] After 5–10 successive deproteinizations, aliquots of the DNA were dissolved in 1 ml CsCl (pH 7.5, 1.80 gcm^{-3}) and overlayered with 1 ml each of a CsCl solution at 1.7 and 1.6 $gcm.^{-3}$ After centrifugation at 36,000 rpm in the Spinco SW 39 for 36 hr to separate DNA from any lingering RNA contamination, the tube was pierced, and the highly viscous DNA fractions were collected. The pooled DNA fractions were then dialyzed for 8 hr against three changes of cold dilute saline citrate buffer. The melting profile of the DNA showed a T_m of 70–71°C (in dilute saline citrate buffer) and a hyperchromicity of 37%.

Hybrid formation and determination: The annealing conditions used were those described by Scherrer *et al.*[16] Routinely, 50 μg DNA and 2–4 μg P^{32}-labeled RNA were used. For the formation of complex, incubations of 2–3 hr at 63°C and of 24 hr at 42°C were equally effective, and saturating values were reached in both cases. The RNA-DNA mixture was brought to a density of 1.81 gcm^{-3} by the addition of CsCl, transferred to a Spinco SW 39 centrifuge tube, and overlayered with more CsCl solution of the same density to a volume of 1.5 ml. The solution was then overlayered with 1.5 ml of CsCl solution (1.63 gcm^{-3}) and 1.5 ml of paraffin oil, and the samples were centrifuged (in the cold) for 72 hr at 33,000 rpm or, alternatively, for 50 hr at 35,000 rpm. The centrifuge tubes were then pierced and five-drop fractions collected. O.D. at 260 mμ and P^{32} radioactivity were determined for each fraction. The fractions containing the hybrid peak were collected, dialyzed against dilute saline citrate buffer, and RNased with 5 μg/ml DNase-free RNase for 1–2 hr at room temperature. The RNase-resistant material was precipitated with 5% TCA in the presence of carrier RNA and its radioactivity determined with a Nuclear-Chicago D-181 micromil window gas flow counting system. The contribution of the RNase-resistant core of the nonhybridized P^{32}-RNA was found to be 4%, and corresponding adjustments in the final values were made.

For preliminary experiments, the filter disk method of Nygaard and Hall[24] was employed. After completion of the annealing process, the RNA-DNA mixture was incubated with RNase 5 μg/ml for 30 min at 30°C. The mixture was then filtered through the membrane filter and washed twice with 5 ml of 0.5 NaCl + 0.01 *M* tris pH 7.4 at 45°C. The results obtained by this method are in general agreement with those obtained by CsCl centrifugation.

Results.—The hybrid between ribosomal RNA and DNA is characterized by its extreme stability toward RNase and its buoyant density which is intermediate between those of DNA and RNA. These two features may be easily demonstrated with hybrids formed between P^{32}-labeled ribosomal RNA from pea cytoplasm and DNA from pea nuclei. Thus, the hybrid is resistant to ribonuclease treatment (Fig. 1). In addition, the hybrid[13] possesses a buoyant density in CsCl significantly higher than that of DNA (Fig. 2). DNA becomes saturated—that is, combines with all of the ribosomal RNA with which it is capable of combining—at a level of 0.17 μg RNA/100 μg DNA in the case of the 28 S RNA subunit and at a level of

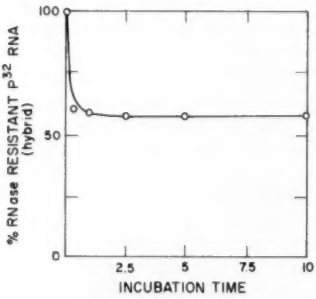

FIG. 1.—RNase resistance of the hybrid between cytoplasmic ribosomal RNA and denatured pea DNA. DNA was annealed with P^{32}-labeled cytoplasmic ribosomal RNA and the hybrid prepared as described in *Materials and Methods*. The hybrid was incubated with 5 μg/ml of DNase-free RNase at 30°C and aliquots were withdrawn and precipitated for counting with 5% TCA in the presence of carrier RNA. Incubation time in hours.

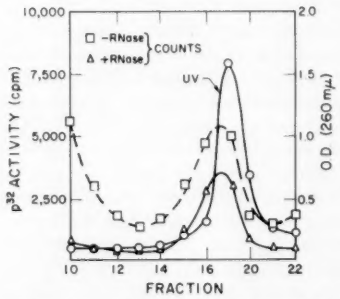

FIG. 2.—CsCl density gradient analysis of the DNA-RNA hybrid. Denatured pea DNA was annealed with P^{32}-labeled cytoplasmic ribosomal RNA and the product centrifuged in a CsCl density gradient until equilibrium had been attained. Centrifugation and determination of radioactivity and absorbancy were carried out as described in *Materials and Methods*. The peak of labeled RNA is shifted to a density higher than that of the DNA.

0.30 μg/100 μg DNA for the combined 28 and 18 S RNA fractions (Fig. 3). The amount of ribosomal RNA complexed per unit DNA is thus similar to that found in bacterial systems.[12–14]

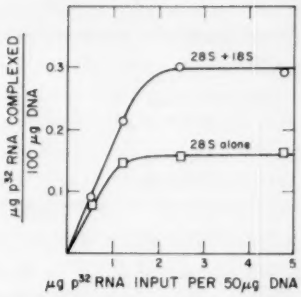

FIG. 3.—Saturation curves for hybrid formation between denatured pea DNA and ribosomal RNA subunits. In each case, 50 μg of DNA were annealed with varying amounts of P^{32}-labeled RNA and the hybrids assayed as described in *Materials and Methods*.

The following measures were taken to ensure no appreciable interference by informational RNA. In all experiments the time of incubation of tissue in P^{32} was sufficiently lengthy (24–36 hr) to permit uniform labeling of all cellular RNA.[25] At the end of the incubation, the bulk of the radioactivity therefore resides in the ribosomal RNA fraction which accounts for approximately 80 per cent of the total RNA. In addition, after the incubation with P^{32}, a large excess (10,000-fold) of unlabeled orthophosphate was added, and the seedlings were incubated for an additional 12 hr. This provides time for the turnover of unstable RNA. Selection against messenger RNA also occurs during preparation of the 80 S ribosomes and the subsequent repetitive sucrose gradient centrifugations of the deproteinized RNA in which only the 28 and 18 S peak fractions were collected. We find, too, that the hybrid between P^{32}-labeled ribosomal RNA and DNA has a buoyant density higher than that of DNA. This is in agreement with the findings of Yanofsky and Spiegelman[13] who have shown that the hybrid between *E. coli* ribosomal RNA and *E. coli* DNA is denser than is DNA. This is not true of hybrids between informational RNA and DNA whose density coincides with that of DNA,[13] due perhaps to complexing of only a very small portion of any given DNA molecule with its complementary RNA.

A fourfold excess of *E. coli* ribosomal RNA does not interfere with the annealing of P^{32}-labeled pea ribosomal RNA to pea DNA. This confirms previous findings that heterologous RNA's do not compete for the same DNA since hybrid formation is base sequence specific. There is, as we would expect, competition between P^{32}-labeled and unlabeled pea cytoplasmic ribosomal RNA for pea DNA. The amount of P^{32}-labeled RNA complexed is inversely proportional to the input of unlabeled RNA (Table 1). Nucleolar (ribosomal) RNA prepared from the whole

TABLE 1

SATURATION OF DENATURED NUCLEAR AND NUCLEOLAR PEA DNA BY RNA OF RIBOSOMAL SUBUNITS

DNA source	P^{32} Pea RNA	P^{31} RNA source	μg P^{32}RNA/ 100 μg DNA
Pea nucleus	28 + 18 S	. . .	0.29
T4	28 + 18 S	. . .	0.02
Pea nucleus	28 + 18 S	*E. coli* ribosomal RNA (4×)	0.31
Pea nucleus	28 + 18 S	Pea cytoplasmic ribosomal RNA (1×)	0.16
Pea nucleus	28 + 18 S	Pea cytoplasmic ribosomal RNA (4×)	0.08
Pea nucleus	28 + 18 S	Pea nucleolar 2*M* NaCl precipitable RNA (4×)	0.10
Pea nucleus	28 + 18 S	Pea nucleolar total RNA (4×)	0.13

Values were determined from saturation curves as in Fig. 2.

TABLE 2

HYBRID FORMATION BETWEEN DENATURED PEA DNA AND HETEROLOGOUS AND HOMOLOGOUS RNA'S

DNA source	P^{32} RNA	μg P^{32} RNA/100 μg DNA
Nucleus	28 + 18 S	0.30
Nucleus	28 S alone	0.17
	18 S (by difference)	0.13
Nucleoli	28 + 18 S	0.32
Nucleoli	28 S alone	0.19

Annealing procedure and hybrid assay as described in *Materials and Methods*.

nucleolar RNA by 2 *M* NaCl precipitation[27-29] also competes effectively with P^{32}-labeled ribosomal RNA in complex formation. Whole nucleolar RNA is somewhat less effective in this function since about half of it is low-molecular-weight RNA (Fig. 4) identified as transfer RNA[26] by its capacity to engage in aminoacyl RNA formation. That the RNA extractable from the nucleolus is not derived from contamination by cytoplasmic ribosomes has been previously shown.[6]

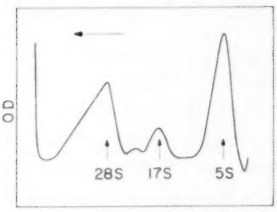

FIG. 4.—Spinco Model E analysis of nucleolar RNA. The RNA was extracted as described earlier.[6] Centrifugation was carried out according to Vinograd *et al.*[20] 0.015 ml of an RNA solution (150 μg/ml) were overlayered on 2 *M* CsCl (pH 7.0) and centrifuged at 56,000 rpm at 13.8°C. For determination of the sedimentation values ($S_{w,20}$), the RNA samples were analyzed by standard UV-boundary centrifugation and the sedimentation values thus obtained applied to the UV pattern of the band centrifugation. The band centrifugation, which allows rapid analysis accompanied by high resolution, of small quantities of material was used routinely to determine the contribution of the different RNA species to the total O.D. of the nucleolar extract.

The 28 and 18 S RNA represent 50–60% of the extractable nucleolar RNA. A minor RNase-sensitive component with a sedimentation constant of 23 $S_{w,20}$ (by linear intrapolation) was found in all experiments. Occasionally, but not reproducibly, a 35–40 S component contributing 5–10% of the total O.D. was also recovered in the nucleolar RNA preparation.

DNA obtained from nucleus-free nucleolar preparations also complexes with cytoplasmic ribosomal RNA and reaches saturation at levels only slightly higher than those characteristic of the whole nuclear DNA (Table 2). The DNA stretches complementary to cytoplasmic (and hence also to nucleolar) ribosomal RNA are therefore not concentrated in the nucleolus but are in fact predominantly in the chromatin of the extranucleolar nucleoplasm.

Discussion.—We have shown that P^{32}-labeled pea ribosomal RNA hybridizes with the whole genomal DNA of pea seedlings to form an RNA–DNA hybrid. This hybrid possesses a buoyant density characteristically higher than that of DNA and is resistant to RNase. The pea ribosomal RNA does not hybridize with DNA of heterologous origin. Heterologous RNA does not interfere with complex formation between pea ribosomal RNA and pea DNA. The complex formed between P^{32}-labeled ribosomal RNA and pea DNA can therefore be attributed to base sequence complementarity between ribosomal RNA and particular stretches of the genomal DNA.

Nucleolar RNA competes with pea ribosomal RNA for the DNA stretches complementary to the latter. This behavior is accentuated if the nucleolar 18 and 28 S RNA components are freed of transfer RNA by precipitation of the higher-molecular-weight material in 2 *M* NaCl.[22-24] We conclude from this fact that nucleolar RNA is not only similar to cytoplasmic ribosomal RNA in base composition[5] and sedimentation behavior,[6] but also in base sequence. Thus, nucleolar RNA includes ribosomal RNA.

The fraction of the genomal DNA capable of com-

plexing with ribosomal RNA, 0.3 per cent, indicates that in the nucleus, as in bacteria, there is a multiplicity of DNA stretches complementary to ribosomal RNA.

Since some 10–15 per cent of the nuclear DNA is intimately associated with the nucleolus,[6, 22, 30–33] exclusive intranucleolar localization of the DNA complementary to ribosomal RNA would reveal itself by causing nucleolar DNA to become saturated with ribosomal RNA at a level 7–10 times higher than that characteristic of total genomal DNA. Our results show, however, that the nuclear and nucleolar DNA become saturated with ribosomal RNA at approximately the same level. It is clear therefore that while a portion of the cistrons complementary to ribosomal RNA is found in the nucleolus, the great majority of cistrons are not, but are to be found elsewhere in the genome. Base complementarity of DNA to cytoplasmic ribosomal RNA is therefore a characteristic not of the nucleolus, but of the chromatin which may be calculated to contain 90 per cent of all the cistrons complementary to ribosomal RNA and possibly more, since the 10 per cent of the nuclear DNA present in the nucleolar preparations are known to include some random chromatin contamination.[34] The possibility of an exchange and therefore randomization *in situ* between nucleolar and extranucleolar DNA may be dismissed as shown by the experiments of Harries.[35]

Our data suggest that ribosomal RNA is synthesized principally by the extranucleolar chromatin and is only subsequently transferred to the nucleolus. Such transfer of newly synthesized RNA from chromatin to the nucleolus has been reported by Rho and Bonner[2] for pea embryo tissue.

We have previously reported the existence in the nucleolus of a large pool of protein which resembles ribosomal protein in amino acid composition.[31] We have also shown that this protein fraction becomes labeled when whole cells, nuclei,[36] or isolated nucleoli[31] are incubated with labeled amino acids. Movement of protein from nucleolus to extranucleolar chromatin is indicated by pulse-chase experiments *in vivo*[37] and *in vitro*.[30] We conceive of the possibility that ribosomal protein of nucleolar origin complexes with newly synthesized ribosomal RNA on or near the chromatin and that the resulting ribonucleoprotein is then transferred to the nucleolar periphery. The considerable heterogeneity of the nucleolar ribonucleoproteins, the large proportion of subunits present,[6] and their inability to support protein synthesis[6, 36, 38] all suggest that the nucleolar ribosomes are in fact unfinished ribosomal precursors whose accumulation in the nucleolar region may be for the final modification and completion required for production of functional ribosomal units.

Summary.—It has been shown that the structural RNA of cytoplasmic ribosomes of pea seedlings can be hybridized with denaturated pea-seedling DNA. Such hybridization, at RNA saturation, involves 0.3 per cent of the total genomal DNA indicating that a multiplicity of DNA stretches are involved in the production of ribosomal RNA. The RNA of the nucleolus also hybridizes with the DNA stretches which are complementary to ribosomal RNA. Nucleolar RNA would appear therefore to contain ribosomal RNA. Only a minority of ribosomal RNA cistrons occur in the nucleolar DNA while the vast majority is to be found in the chromatin. It is concluded that the bulk of the ribosomal RNA—if not all—is manufactured by nonnucleolar regions of the chromatin and that the ribosomal RNA is then transferred into the nucleolus for final assembly into ribosomes.

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There is in our data (see also ref. 13) an indication that the ribosomal RNA's are diverse. This is the fact that the RNA input required for saturation of DNA is far in excess of the RNA ac-

tually complexed. This could be due to low concentrations of particular ribosomal species in a population of ribosomal RNA. Such a diversity of ribosomal RNA could arise from mutation and would parallel the degeneracy of sRNA.

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A CHARACTERIZATION OF LEXICOGRAPHICALLY ORDERED η_α-SETS

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The sets of the title were introduced by Hausdorff[1]; for the case of groups, see Alling.[2]

By an *initial segment* of a linearly ordered set X we understand a (possibly void) subset which contains with any element all preceding elements; a terminal segment is defined dually. A *cut* of X is a decomposition into an initial and terminal segment; if neither is void, the former has no last, and the latter no first element, we speak of a *gap*.

Let α be a fixed ordinal number. We shall call an initial segment *short* if it contains a cofinal (i.e., generating qua initial segment) well-ordered subset of type $< \omega_\alpha$, otherwise *long;* and dually for terminal segments. An X which admits no cuts into short segments is called an η_α-*set*.

From now on X will be such a subset of the product of the linearly ordered sets X_j over the linearly ordered indexing set J that distinct elements of X differ at a first j; by ordering these elements lexicographically, i.e., in accord with the order of their j^{th} components in X_j for this first j, one makes X into a linearly ordered set. If H is an initial segment of J, we write P_H for the projection of X into[3] $\bigtimes_{h\in H} X_h$; it is monotone. If H consists of all elements preceding some j and $x_H \epsilon P_H(X)$, we call the linearly ordered subset of X_j consisting of j^{th} components of $P_H{}^{-1}(x_H)$ the j^{th} *residue set of* X *corresponding to* x_H.[4]

The initial segments Y of such a subset X have been classified by Hausdorff in the following terms: Let H be the union of all initial segments G of J such that $P_G(Y)$ has a last element x_G. These elements agree with each other over common indices; therefore they determine a unique element $x_H \epsilon \bigtimes_{h\in H} X_h$. Three exhaustive and mutually exclusive possibilities now present themselves: either (*i*) $x_H \epsilon P_H(Y)$: then H has no last element and is isomorphic with a cofinal subset of Y; or $x_H \epsilon P_H(Y)$ and either (*ii*) J-H has a first element j: then a nonvoid initial segment without last element of the j^{th} residue set corresponding to x_H is isomorphic with a cofinal subset of Y; or (*iii*) J-H has no first element: then this set inversely ordered is isomorphic with a cofinal subset of Y.[1] Of course the dual classification holds for terminal

segments. It will be useful to know that $P_H^{-1}(x_H) \subset Y$ in case (*iii*); indeed if $x \in Y$, its segment of agreement with every $y \in Y$ is contained in H; it must therefore differ from a $y \in P_H^{-1}(x_H)$ on an $h \in H$.

Conversely, every $x_H \in P_H(X)$, with the added specification, if J-H has a first element j, of a nonvoid initial segment without last element in the corresponding j^{th} residue set, determines an initial segment of type (*ii*) or (*iii*), respectively. Unfortunately, an x_H whose restriction to every proper initial segment G of H belongs to $P_G(X)$ need not be determined by the segment it dominates over H. To minimize the complications, inessential for the purpose in view, arising from this circumstance, it will henceforth be assumed that *no residue set has a first or last element*. Now if Y is the initial segment of X of elements dominated over G by the restriction x_G of x_H, then $x_G \in P_G(Y)$; for otherwise the h^{th} residue corresponding to the restriction of x_H would have a first element for every $h \in H$-G. It follows that Y determines x_H except possibly on a last index of H. The same is valid for the dual terminal segment.

With this preparation one need only enumerate the possible types of complementary initial and terminal segments to obtain the

THEOREM. *Suppose no residue of X has a first or last element. Then necessary and sufficient conditions that X be η_α are:*

(I) *If H is a short initial segment of J without last element and every proper restriction of x_H to an initial segment G belongs to $P_G(X)$, then $x_H \in P_H(X)$.*

(II) *No gap in a residue set has both segments short.*

(III) *If the j^{th} residue corresponding to x_H is a short initial segment, or if J-H has no first element and is short, then: if H has no last element it is long; if h is its last element and x_h its h^{th} component, then either the terminal segment (in the corresponding h^{th} residue) of successors of x_h is long, or x_h has an immediate successor in this residue and the j^{th} residue it defines is a long terminal segment. The dual condition, obtained by interchanging "initial" and "terminal" and replacing "successor" by "predecessor" is to hold as well.*

The result becomes somewhat simpler if one makes the additional assumption that no segment of a residue set is long. Then (II) goes over into the nonexistence of gaps, that is, into the conditional completeness of every residue set; whereas in (III), the first hypothesis is automatically fulfilled, while the conclusion is simply that H is long: in other words, (III) asserts that J is an η_α-set. In this form the theorem is seen to contain Alling's characterization of abelian η_α-groups[2] (which is thus shown to be independent of groups). It is only necessary to remark that all j^{th} residue sets are now isomorphic, being cosets of the one corresponding to $x_H = 0$, which is a nontrivial real subgroup, hence has no short segment if $\alpha > 0$.

The author would like to thank his colleague, Professor Norman Alling, for the stimulating conversations which aroused his interest in this subject.

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*ANALYSIS OF ULTRAVIOLET LIGHT-INDUCED MUTAGENESIS BY DNA TRANSFORMATION IN BACILLUS SUBTILIS**

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During the past decade ultraviolet light-induced mutagenesis has emerged as a process involving metabolic steps of measurable temporal duration. Mutation frequency decline (MFD) occurs when postirradiation nutrient conditions are unfavorable to RNA and protein biosynthesis.[1–5] Conversely, postirradiation conditions conducive to synthesis of RNA and protein encourage stable genomic establishment of the mutations.[2] This irreversible event has been termed mutation fixation (MF).[1] Postirradiation synthesis of DNA is clearly the *terminal* event in the mutation process.[3, 4, 6] The physicochemical nature of the "promutant" state intervening between UV irradiation and MF has not been clarified, and the nature of the *primary* premutational lesion and its relationship with MFD are controversial. Witkin[3, 7] favors DNA as the primary site of UV genetic damage, and considers this damage accessible to repair processes during the "sensitive" period. Haas and Doudney[8] have implicated UV-modified nucleic acid precursors. Inquiries aimed at the *in vivo* relationship of cellular macromolecules and the promutant state are difficult to approach; however, the intracellular localization of the premutational lesion can be studied with certain genetic exchange systems. Kada and Marcovich,[9] using the *Escherichia coli* K12 conjugal system in which the F^- recipient cell received an irradiated *HFr* chromosome, concluded that UV-induced mutations maintain cytoplasmic residence preceding stabilization by chromosomal integration. This communication reports experiments where the mutation assay was restricted to changes localized in the DNA, rather than to more general determinations involving the over-all potential of the entire cell.

Materials and Methods.—Bacterial strains: *Bacillus subtilis* strain 168, requiring indole, and its prototroph, 168-wild, were obtained from S. Zamenhof. Strain S10 requiring glutamic acid (*glu*) was obtained from I. Takahashi.[10] SB25 requires histidine (*his*) and indole (*ind*); SB32 requires *his* and was obtained by the cross 168-wild × SB25. The SB strains were obtained from the Nester-Lederberg collection.[11] Stocks were maintained on potato agar[12] at room temperature. Basal salts for "minimal," "transformation," and "growth" media were those of Anagnostopoulos and Spizizen.[13] Liquid medium for preirradiation growth of SB25 was glucose-basal salts minimal supplemented with 50 μg/ml L-histidine and 30 μg/ml L-tryptophan. Postirradiation medium was the same but with acid-hydrolyzed casein added to a final concentration of 0.2%. Hard agar and soft agar media contained 1.5% and 0.7% Bacto agar (Difco), respectively. Nutrient agar (Difco) served whenever complete medium was needed. A minimal salts-glucose agar, supplemented when required with 30 μg/ml of the appropriate amino acids, was used for selective plating. "Mutation expression" medium was the appropriate selective agar medium containing 0.008% (w/v) dehydrated nutrient broth. Minimal medium was used for serial dilutions, and vigorous shaking of dilutions was essential for consistency as to the number of viable plating units.

Spore suspensions: Cells were grown in potato extract medium and incubated with shaking at 37°C for 72 hr. The culture was centrifuged, washed twice, and resuspended in sterile water. This suspension was heated 30 min in a 70°C waterbath, titrated, and stored at 4°C. Equal volume resuspensions yielded about 5×10^8 spores/ml.

Determination of spontaneous mutation rates: Individual cultures at stationary growth phase vary widely in mutant levels.[14] For this reason spore suspensions were assayed for revertants.

Those with less than 5×10^{-9} revertants/ml were reserved for inoculum in determination of spontaneous mutation rates. The mutation rate per bacterium per division cycle was calculated utilizing Newcombe's formula[15] and Matney's method.[16] Briefly, replicate populations were impinged on 0.45-μ Millipore filter disks, incubated on complete medium for different times, and successively larger populations corresponding to longer incubation times were transferred to selective medium for 48 hr of incubation at 37°C. The time interval (t_2–t_1) was always taken during the logarithmic growth phase. Induced mutation frequency determinations (total mutants/total cell count) were made using the same technique.

Ultraviolet irradiation: The ultraviolet source was a Hanovia model 36,000 mercury vapor arc with an output at wavelengths below 2,800 Å of 40 ergs/mm²/sec. Under our experimental conditions, 20-sec exposures gave 50% survival of cells on the surface of membrane filters. Postirradiation operations were carried out under yellow light.

Chemical determinations: Cell pellets were washed twice with cold 10% trichloracetic acid (TCA). Nucleic acids were hydrolyzed in 10% TCA for 50 min at 70°C, then DNA was analyzed by the diphenylamine reaction of Burton,[17] RNA by the orcinol reaction of Hurlbert *et al.*,[18] and protein by the Folin method of Lowry *et al.*[19] Log phase *Bacillus subtilis* cells at an OD^{660} of 0.20 in glucose minimal salts supplemented with 0.1% casein hydrolysate (1.5×10^8 cells/ml) yield about 175 μg/ml protein, 8 μg/ml DNA, and 70 μg/ml RNA.

Preparation of strain SB25 DNA used for "selfing": Samples were taken at various postirradiation incubation times, and the volumes manipulated so as to yield approximately equal cell numbers. These were held at 0°C until centrifuging at 5,000 × *g* for 10 min at 4°C. Pellets were resuspended in 4 ml of 0.05 *M* sodium citrate/0.015 *M* sodium chloride and incubated at 37°C with 50 μg/ml of lysozyme for 30 min. Four ml of 20% (w/v) sodium chloride were added to each tube, and incubation was continued for 30 min. Two per cent sodium desoxycholate (w/v) was added at 1/20 volume,[13] and incubation with gentle shaking continued for 1 hr preliminary to overnight storage at 4°C. Precipitates were removed by centrifugation at 5,000 × *g* for 10 min, and supernatants added to 2 vol of ethyl alcohol; precipitating DNA was harvested and redissolved in 0.015 *M* sodium chloride. An equal volume of 20 *M* sodium chloride was added to make a final concentration of about 10%. This was used as the transforming preparation after adjusting its DNA concentration to 2–5 μg/ml. Postirradiation DNA samples were stored at 4°C under a drop of chloroform.

Pre- and postirradiation cultural conditions: Spores of SB25 were inoculated into 20 ml of growth medium and incubated at 37°C overnight. This culture was filtered, and the cells were resuspended with vigorous shaking in 500 ml of warm preirradiation minimal medium. At an OD^{660} indicating 1.0×10^8 cells/ml, 15-ml aliquots were filtered. The filter-impinged cells were placed on the surface of cold soft agar plates at 4–6°C, irradiated, and then eluted from the filters into prewarmed postirradiation medium. This was considered the zero point of postirradiation time. Samples were taken for OD^{660} readings, viable count platings, DNA extractions, and chemical determinations at suitable intervals of incubation.

Assay for mutation transformants: DNA to be assayed was added to thawed SB25 recipient cells which were then incubated for 30 min at 37°C on the shaker. Suitable dilutions were filtered and the cells impinged on 0.45-μ filter disks. These were incubated on moist nutrient agar plates for 3 hr. The disks were then transferred to minimal soft agar for 10 min to reduce nutrient carry-over, and then placed on "expression medium" agar for 48 hr at 37°C.[20] Several identical experiments were performed using the same batch of recipient cells and the same donor DNA preparation.[21]

Extraction of "nonextractable DNA": The protein-DNA-detergent pellet was dialyzed for 48 hr against 0.015 *M* sodium chloride/0.05 *M* sodium citrate. A solution containing 50 μg/ml trypsin, 25 μg/ml chymotrypsin, and 0.005 *M* $CaCl_2$ was added, and the cells were incubated with shaking for 3 hr at 37°C. The pellet was again dialyzed for 72 hr. Sodium chloride was added to a final concentration of 0.15 *M*, and the solution slowly stirred at 4°C overnight. This solution was added slowly to 2 vol of ethyl alcohol, centrifuged at 6,000 × *g* for 10 min, and the pellet then dissolved in DNA solvent. This preparation was stored at 4°C under chloroform. Total DNA and RNA were determined by hydrolysis of cell samples; nonextractable DNA was determined as the difference between the colorimetric assay of total DNA and extractable DNA (Fig. 1). Methods for normal DNA extraction, preparation of recipient cells, and their maintenance have been described.[20–22]

Results.—The postirradiation kinetics of mutation were followed using the transforming activity of DNA, extracted from irradiated auxotrophic cells of strain SB25, for isogenic unirradiated recipient cells of the same strain. The mutations *ind*$^-$ → *ind*$^+$ and *his*$^-$ → *his*$^+$ were scored, and such mutations were detected to the extent that they could compete with nonmutated loci as donors in the transforming system. Since the frequency of induced mutants donating either *ind*$^+$ or *his*$^+$ to recipient cells might have been *less* than the occurrence of spontaneous reversions, it was necessary to determine spontaneous mutation frequencies. Table 1 gives the spontaneous revertant frequencies and indicates that these do not increase in response to nutrient supplementation. However, the UV-induced mutation frequencies for both mutations increase considerably with nutrient broth (NB) supplementation. UV-induced mutant yields are high even without supplementation due to the initial 2-hr incubation on complete medium. Spontaneous mutation frequencies for the homologous single auxotrophs of SB25 are presented in Table 2.

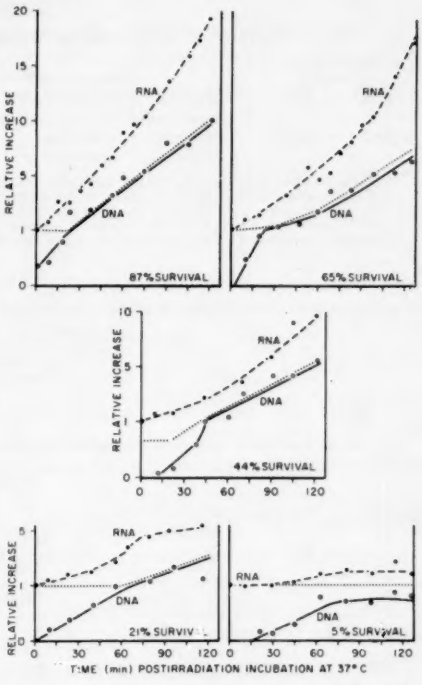

FIG. 1.—Postirradiation nucleic acid synthesis. Postirradiation cultures from various UV survival levels were assayed periodically by colorimetric methods for total DNA and RNA (dotted lines). At the same times, samples were taken for DNA extraction which was assayed chemically (solid lines). The difference between the total DNA and extractable DNA represents "nonextractable DNA" (see text).

Determination of UV-induced mutation frequency: Detection of a "mutation transformant" requires occurrence of both a mutation and a transformation, and the sensitivity of the assay increases in proportion to increases in these frequencies. Initial experiments were therefore directed to achieving high mutation frequencies. Induced reversion frequencies at a UV dose effecting 65 per cent survival of the irradiated cells are presented in Table 2. Reversion of *his*$^-$ → his$^+$ is relatively more sensitive to UV than reversion of *ind*$^-$ → ind$^+$; but *his*$^-$ is less stable than *ind*$^-$ to reversion whether the mutation is spontaneous or UV-induced.[23] Mutant yields obtained under various postirradiation nutritional conditions are summarized in Table 3. Both single mutations increase in frequency under "step-up" nutrition, and both exhibit MFD in the presence of chloramphenicol or under starvation.

UV effect upon DNA extractability: Figure 1 illustrates a UV dose-dependent decrease in DNA extractability when the usual methods of extraction are used. This loss recovers with incubation, and recovery is usually complete when postirradiation DNA synthesis begins. Smith[24] proposes that nonextractable DNA is noncovalently associated with a protein moiety due to UV treatment. If this is

TABLE 1

EFFECT OF NUTRITION ON MUTATION IN *B. subtilis* STRAIN SB25

Prototrophic mutation	Selective medium	Mutation Frequency: Spontaneous	Mutation Frequency: UV-induced
His$^-$ → *His*$^+$	Minimal + tryptophan*	1.3×10^{-7}	5.0×10^{-4}
	Minimal + tryptophan + 1% NB	1.4×10^{-7}	3.5×10^{-3}
Ind$^-$ → *Ind*$^+$	Minimal + histidine	8.7×10^{-7}	3.0×10^{-5}
	Minimal + histidine + 1% NB	8.5×10^{-7}	2.0×10^{-4}

* Tryptophan was used to supply the indole requirement of strain SB25.
Ind, denotes indole; *His*, histidine; NB, nutrient broth (Difco).
A dose of ultraviolet allowing *ca.* 50% survival was used for inducing mutations in cells impinged on filter disks. Irradiated and nonirradiated bacteria (on filter disks) were incubated 2 hr on a complete medium, transferred to appropriate mutation expression (selective) medium, and incubated for 2 days.

TABLE 2

COMPARISON OF SPONTANEOUS AND ULTRAVIOLET-INDUCED MUTATION FREQUENCIES IN *B. subtilis*

Strain	Spontaneous Reversions (× 10^{-8}): *Ind*$^+$	*His*$^+$	Ratio: *Ind*$^+$/*His*$^+$	Ultraviolet-induced Reversions (× 10^{-8}): *Ind*$^+$	*His*$^+$	Ratio: *Ind*$^+$/*His*$^+$
168	2.3	—	—	50	—	—
SB32	—	6.9	0.3	—	3200	0.02

See text for method of determining mutation frequencies.

TABLE 3

EFFECT OF POSTIRRADIATION NUTRITION ON UV-INDUCED MUTATION FREQUENCIES IN *B. subtilis*

	Postirradiation nutrition	*His*$^-$ → *His*$^+$ mutation, Postirradiation Incubation Time, 0 min	25 min	65 min	*Ind*$^-$ → *Ind*$^+$ mutation, Postirradiation Incubation Time, 0 min	25 min	65 min
		*Mutation frequencies × 10^{-7}					
Strain SB25	Step-up medium	43	480	1,010	9	24	110
	CM medium	49	12	12	14	52	31
	Starvation medium	77	34	20	12	64	20
Strain 168	Step-up medium	...	...	...	5.9	13.0	85.0
	CM medium	...	...	...	3.3	0.1	0.1
	Starvation medium	...	...	...	4.3	0.9	0.4
Strain SB32	Step-up medium	230	1,390	4,310	...	...	...
	CM medium	120	10	4	...	...	...
	Starvation medium	170	39	20	...	...	...

* See text for method of determining mutation frequencies.
Immediately after ultraviolet irradiation, cells were resuspended in "step-up" medium (see *Materials and Methods*), "step-up" medium containing 50 μg/ml chloramphenicol (CM medium), or in minimal medium lacking all amino acids (starvation medium). Incubation was continued and cells were plated at indicated times on appropriate "mutation expression" media.

true, the DNA-protein-detergent pellet might be amenable to extraction treatments using aqueous solvents. The pellet was dialyzed (see *Materials and Methods*), treated with trypsin and chymotrypsin, and redialyzed. The alcohol precipitate was dissolved in 10 per cent NaCl and tested for ability to transform *glu*$^-$ auxotrophs to prototrophs [i.e., UV-SB25 (*his*$^-$ *ind*$^-$ *glu*$^+$) × S10 (*his*$^+$ *ind*$^+$ *glu*$^-$)]. Results of this experiment indicated that this DNA can serve as transforming principle for *glu*$^+$, although the efficiency of UV-treated DNA was about one fourth that of the unirradiated control (strain 168-wild). No mutations to *his*$^+$ or *ind*$^+$ were scored in the cross: UV-SB25 (*his*$^-$ *ind*$^-$) × SB25 (*his*$^-$ *ind*$^-$). It thus appears that DNA extracted from cells early after irradiation possesses few mutations in either the extractable or nonextractable DNA. This conclusion is substantiated by results shown in Figure 1 at the 65 per cent survival level. At this UV dose, previously nonextractable DNA has become extractable *before* the onset of DNA synthesis. Nevertheless, no *ind*$^+$ or *his*$^+$ mutation transformants were detected until well after DNA synthesis was resumed. The yields of mutation transformants corre-

TABLE 4

DNA-MEDIATED MUTATION TRANSFORMANTS (TRANSFORMANTS/10^8 ORGANISMS)

UV survivor level	Prototroph scored	Postirradiation time of DNA extraction (min) 5	20	35	50	65	80	95	110	125
	Histidine	0	0	58	61	70	63	63	70	69
87%	Indole	0	0	2	0	2	0	0	0	0
			↓1	↓2						
	Histidine	0	7	260	392	401	399	351	400	371
65%	Indole	1	6	38	64	60	58	66	54	50
			↓1		↓2					
	Histidine	1	3	710	4324	4418	4107	4301	3939	4190
44%	Indole	0	4	54	198	203	187	176	171	209
			↓1		↓2					
	Histidine	6	3	12	4	22	38	43	40	43
21%	Indole	7	0	9	7	8	13	12	1	4
						↓1	↓2			
	Histidine	11	13	10	9	10	13	11	14	8
5%	Indole	19	15	10	19	20	13	18	15	20

Arrows designated "1" and "2" indicate the approximate time when postirradiation DNA synthesis begins and when DNA doubles, respectively.

sponding to the data presented in Figure 1 are given in Table 4. It is striking that few mutation transformants precede postirradiation resumption of DNA synthesis. Furthermore, there is little increase in mutation frequency after the DNA has doubled. Virtually the entire crop of UV-induced mutations appears in DNA extracted during the interval between postirradiation resumption of DNA synthesis and doubling of postirradiation DNA. UV doses allowing about 50 per cent survival produced optimum mutation transformant yields, and higher or lower doses were less effective. The kinetic pattern for histidine reversion is qualitatively similar to that of indole reversion, but *his*$^+$ mutations occur at substantially greater frequencies than *ind*$^+$ mutations, and the ratio varies with UV dose.

Effect of chloramphenicol: DNA was extracted during postirradiation incubation conditions that promoted MFD, i.e., chloramphenicol (CM) challenge.[1] When 25 μg/ml CM was added 2 min after UV irradiation, no mutation transformants were recovered at any postirradiation time. If the mutagenic site of UV action were the DNA, and if chloramphenicol allowed UV-lesion repair processes to occur,[3, 7] then these conditions should promote *maximum* recovery of mutations in DNA extracted at earlier postirradiation times before repair of the mutation lesions could occur.

Since nonextractable DNA was at a maximum at this early time, experiments were performed similar to that summarized in Table 4 for the 44 per cent survival level, but the postirradiation sample was divided into three parts. One portion was incubated as a control and treated in the manner previously described. Second and third portions were treated with 25 μg/ml CM after 20- and 25-min postirradiation incubation, respectively. DNA was extracted at various intervals and tested for *ind*$^+$ and *his*$^+$ mutations. Results are presented in Table 5. CM treatment clearly promoted MFD, and only mutations "fixed" in the DNA at the time of CM addition were maintained. Twenty-five min after irradiation, DNA synthesis in the control sample resumed with attendant progressive increase in resident mutations. CM addition at this time arrested increase of mutations in transforming DNA subsequently extracted. However, CM challenge at 20 min after irradiation (DNA

TABLE 5

EFFECT OF MUTATION FREQUENCY DECLINE ON MUTATION TRANSFORMING ACTIVITY IN *B. subtilis*

Postirradiation incubation condition	Postirradiation Time of Donor DNA Extraction (min)							
	5	20	30	35	45	60	75	90
	Histidine prototrophs/ml of recipient culture							
Control culture (no CM challenge)	1	6	107	575	1001	991	984	1008
CM challenge at 25 min	0	2	86	94	70	81	81	109
CM challenge at 20 min	1	8	19	17	29	14	14	18

synthesis is just resuming and nonextractable DNA has recovered) markedly reduces mutations in the transforming DNA. Therefore, MFD occurs when the previously nonextractable DNA has become completely extractable.

Recipient cell integration of early postirradiation DNA: The possibility was considered that early postirradiation DNA was not subject to normal integration by recipient cells. To test this, donor DNA's were extracted from SB32 (*his*$^-$ *ind*$^+$) and 168 (*his*$^+$ *ind*$^-$) at 10 min and at 100 min after irradiation. These were used to transform unirradiated SB25 (*his*$^-$ *ind*$^-$) recipients. By virtue of the close linkage between *his* and *ind* (about 75 per cent),[11] a low frequency of mutation transformants should be accompanied by a correspondingly low transformation frequency of the wild marker if integration of the DNA's were inefficient. However, the wild marker was transformed equally well regardless of whether the DNA originated from early or late postirradiation cultures. On the other hand, the mutation transforming activities for either *his*$^+$ or *ind*$^+$ mutations were practically nil in the 10-min DNA's but considerable in those extracted at 100 min. The observed increase of mutation transforming activity in correlation with the time of DNA synthesis must reflect the process of mutation rather than changes in ability of "mutation-bearing" DNA to undergo the transformation process.[25]

Discussion.—The results clearly indicate that the transforming DNA contains no induced mutations until after postirradiation DNA synthesis is initiated. The production of these mutations is confined to the initial postirradiation doubling of DNA. These facts specifically indicate a direct relationship between genic alteration and DNA replication, indicating that UV-induced mutation occurs in daughter DNA but not in parental DNA. The experiments do not substantiate theories favoring parental DNA as the primary site of UV mutagenic action. Witkin[3, 7] proposes a DNA target site where the "lesion" is maintained or repaired depending upon nutritional conditions. Doudney and Young[26] consider DNA to be the most likely primary site of UV action. Such proposals predict that DNA extracted shortly after irradiation would bear the mutant site and should transform an appropriate recipient cell, but this is not the case. The possibility was considered that the promutant state might correspond to a physical-chemical DNA condition refractive to one or more essential steps in the transformation process. UV-inactivated DNA is quite efficiently adsorbed by competent cells.[27] This precludes the possibility that postirradiation DNA extracted early is physically altered so as to prevent its entrance into the cell. It is conceivable that duration of the promutant state is correlated with a physical effect on DNA (such as thymine dimer formation) that results in integration of this DNA being fatal to recipient cells. The methods were sufficiently sensitive to detect a significant frequency of "lethals" occurring in recipients, but none was observed. Another possibility was that the nature of the mutation lesion prior to DNA synthesis simply precludes integration into the

recipient genome. If this were true, then unavailability of promutant DNA sites to the integration process should also exclude a large proportion of closely linked donor sites. However, DNA extracted from early postirradiation cells yields transformation frequencies for wild markers essentially the same as that for DNA extracted late, even though corresponding mutation transformation frequencies differ substantially. It was also possible that nonextractable DNA was causally related to DNA with high mutation content. This possibility was eliminated since (1) nonextractable DNA recovered in an aqueous solvent has high transforming activity for a nonmutated marker, but no transforming activity for the mutations; and (2) at one UV dose level (65 per cent survival), when previously nonextractable DNA has become extractable *before* the onset of DNA synthesis, no mutation transforming activity appeared.

Doudney's hypothesis [26, 28] proposes that the proximal integrity of a "mutation lesion" and a DNA "replication blocking lesion" is requisite to subsequent appearance of the mutation in daughter DNA. If each of these lesions occurs in a separate DNA strand, as suggested, it is possible that the transformation mechanism might disrupt the integrity of the two-stranded structure[29] before DNA is integrated. While Doudney's considerations are plausible, they are not supported by conclusive experiments. We therefore prefer to regress to an earlier hypothesis, namely, that the primary action of UV in mutation induction is production of radio-chemical reactants which enter into cellular interactions producing a pool of modified nucleic acid precursors. The availability of these small mutagenic molecules is subject to metabolic control until their polymerization as stable genic material. Irreversible mutation is effected in some manner by the incorporation of these modified precursors into DNA.[8]

Extracellular phages respond only slightly to the mutagenic action of UV,[30] and direct irradiation of transforming DNA produces few if any mutations.[31] In general, DNA does not seem to be remarkably susceptible to mutation by UV, although DNA synthesis is quite sensitive.[32, 33] Similarly, in these experiments, DNA extracted from *in vivo* cells immediately after UV exposure does not manifest detectable mutation. These considerations suggest that the intracellular site of UV action leading to mutation is primarily extragenomic.

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POLYRIBONUCLEOTIDES AS TEMPLATES FOR POLYDEOXYRIBONUCLEOTIDES

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The mechanism by which DNA transfers its information for the synthesis of both DNA and RNA has not been established. Base analysis and nearest-neighbor frequencies imply that the DNA acts as a template *in vitro*. In the case of the RNA-polymerase reaction *in vitro*, the experimental evidence indicates overwhelmingly that the DNA template remains intact and helical.[1, 2] On the other hand, in DNA synthesis the identity of the template and the product make a similar conclusion less certain. In this paper we show that the helical complex poly (A + U), which is a two-stranded polyribonucleotide, serves as a template for the synthesis of a polydeoxyribonucleotide, poly (dA + T), using DNA polymerase *in vitro*. The poly (dA + T) product has been shown to be an ordered macromole-

cule (probably helical) in which each molecular chain contains only one type of base, like the template poly (A + U). The evidence presented here lends support to the suggestion made some time ago that the parental DNA remains intact during DNA synthesis.[3]

Materials and Methods.—Poly A and poly U were purchased from the Miles Laboratories, New Jersey. Poly (A + U) primer was prepared by mixing equimolar amounts of poly A and poly U in the presence of 10^{-4} *M* Mg^{++}.[4] The sedimentation constants of poly A, poly U, and poly (A+U) in 0.2 *M* sodium chloride were 21.8, 14.1, and 30 Svedbergs, respectively.

C^{14}-labeled deoxynucleoside-5′-triphosphates were obtained from Schwarz BioResearch, Inc. P^{32}-labeled deoxynucleoside-5′-triphosphates were kindly supplied by Dr. F. J. Bollum. Unlabeled deoxynucleoside-5′-triphosphates were purchased from Pabst Laboratories. Spleen phosphodiesterase was purchased from Worthington Biochemical Corporation. Micrococcal DNase was a gift from Dr. Charles Dekker.

DNA polymerase was prepared from *E. coli B*. The bacteria were grown with vigorous aeration at 37°C. The medium contained, per liter, 7 gm of K_2HPO_4, 3 gm of KH_2PO_4, 0.05 gm of Na-citrate·$2H_2O$, 0.1 gm of $MgSO_4$ (anhyd.), 1 gm of $(NH_4)_2SO_4$, 6 gm of yeast extract, and 10 gm of dextrose. The culture was chilled and harvested at the end of log phase. The cells were collected and washed by suspension in 0.5% NaCl–0.5% KCl, centrifuged, and then stored at −20°C.

DNA polymerase was prepared by the method of Furth, Hurwitz, and Anders.[5] The purification results are shown in Table 1.

TABLE 1

PURIFICATION OF DNA POLYMERASE

Step	Total units	Protein, mg/ml*	Specific activity†
Crude	21,000	56.44	1.07
DEAE cellulose‡	5,000	0.205	2,500

* Protein was determined by the procedure of Lowry *et al.*[6] Before the determination, the enzyme solution was dialyzed against 0.005 *M* Tris, pH 8.4.

† Enzyme activity was measured by following the incorporation of C^{14}-labeled deoxynucleoside-5′-triphosphates into acid-insoluble product. Enzyme activity is expressed as units per milligram of protein. A unit of enzyme is defined as that amount of enzyme which catalyzes the incorporation of 1 mμmole of labeled substrates into an acid-insoluble product in 20 min at 37°C.

‡ Before column chromatography, the DNA polymerase was separated from RNA polymerase by fractional precipitation with $(NH_4)_2SO_4$. No RNA polymerase activity was demonstrable in the purified DNA polymerase fraction when the ribonucleoside-5′-triphosphates were used as substrates with a DNA primer.

The course of polymer synthesis was followed both by incorporation of P^{32}-labeled deoxyribonucleoside-5′-triphosphates and, more frequently, by the hypochromic shift which accompanies the conversion of the substrates into an ordered polymer. The reaction was stopped by cooling and adding sodium chloride to a final concentration of 0.2 or 0.4 *M*. In some experiments, the enzymes also were inactivated by the addition of duponol to a final concentration of 1%. Helix-coil transitions and sedimentation velocity measurements were then carried out in its presence, after removal of the excess substrates by dialysis. In these cases the ratio of product to primer varied from 10 to 20. In other experiments, deproteinization was effected by shaking with chloroform-octanol in the cold. The solutions were then dialyzed in the cold against 0.2 *M* NaCl, H_2O, and finally against 0.02 *M* NaCl. The product was obtained by lyophilization or by precipitation with three volumes of cold ethanol.

The enzymatic hydrolysis of the product to 3′-deoxyribonucleotides by micrococcal DNase and spleen phosphodiesterase was carried out according to the method of Josse, Kaiser, and Kornberg.[7] The nucleotide analyses were carried out on Dowex-1 formate columns.[8] The results are given in Table 2. In each case the synthesis was carried by reacting 0.5 μmoles each of dATP and TTP in 1 ml of solution containing 0.46 optical density units of poly (A + U) and about 10 units of polymerase. The solution was 0.005 *M* in magnesium chloride, 0.05 *M* in Tris, pH 8.4, and 0.001 *M* in mercaptoethanol. The reaction was terminated after 20 min. The product was isolated as described by Hurwitz *et al.*[1] and hydrolyzed with micrococcal DNase followed by spleen phosphodiesterase.

Sedimentation velocity and CsCl density gradient measurements were made with a Spinco Model E ultracentrifuge equipped with ultraviolet absorption optics.

Light-scattering measurements were made in 0.2 *M* NaCl with a Brice-Phoenix universal light-scattering photometer. The poly (dA + T) solution was clarified by centrifuging for one-half hour at 30,000 × *g*, after which the top two thirds of the contents of the tube was removed and transferred to a dust-free conical cell. The solvent was cleaned by repeated filtration through a 0.45 μ Millipore filter. Turbidities were calculated for angles between 20° and 135°.

Helix-coil transitions were determined in a Beckman Model DU spectrophotometer equipped with a thermostatically controlled cell compartment.[9]

Results.—(1) *Synthesis of poly (dA + T) primed by poly (A + U):* Figure 1 shows the course of both an unprimed and a poly (A + U) primed reaction. It can be seen that there is a 7-hr lag period for the unprimed poly (dA + T) synthesis, while in the primed synthesis the induction period is markedly reduced and the maximum decrease in optical density at 260 mμ is found at 25 min.

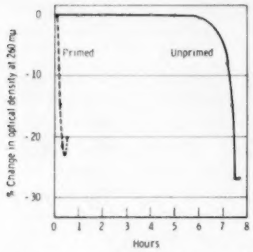

FIG. 1.—Comparison of poly (A + U) primed versus unprimed synthesis of poly dAT. The composition of the mixture was: 5×10^{-2} *M* Tris, pH 8.4; 5×10^{-3} *M* $MgCl_2$; 1×10^{-3} *M* mercaptoethanol. Each milliliter contained 0.5 μmoles each of dATP and TTP. The primed reaction contained 0.34 optical density units of poly (A + U).

(2) *Nearest-neighbor analysis of the product:* In order to determine that the poly (dA + T) was in fact homopolymeric in each chain just as the primer is, a nearest-neighbor analysis was carried out. Two reaction mixtures were studied: one contained dATP and $TP^{32}PP$ and the other, $dAP^{32}PP$ and TTP. The results are contained in Table 2, where it is shown that, within experimental error, each adenylate is followed by an adenylate in the product and each thymidylate, by a thymidylate.

(3) *Physical properties of poly (dA + T):* (a) *Ultracentrifugation:* Figure 2 shows sedimentation velocity patterns of a typical preparation of poly (dA + T) which had been primed by poly (A + U). The sedimentation constant calculated from this run is 28 S, although different values were obtained for the various preparations. The range was 18–30 S. The variation is due principally to the size of the primer and the quantity of nucleases present in the polymerase used. The poly (dA + T) was found to be extremely sensitive to nuclease action, and in a number of instances the product was degraded to a very low molecular weight. Another cause for the variation in the molecular weight and sedimentation constant is the degree of aggregation. In one instance the molecular weight was decreased from 6×10^6 to 1.8×10^6 on further deproteinization by shaking with chloroform-octanol. The boundary shown in Figure 2 is quite sharp, indicating a narrow distribution of sedimenting species and implying a narrow molecular weight distribution.

The density determined in a cesium chloride density gradient is 1.674 ± 0.005.

TABLE 2

DETERMINATION OF NUCLEOTIDE SEQUENCE IN POLY (dA + T) PRIMED BY POLY (A + U)

		3′-deoxyadenylate		3′-thymidylate	
Experiment	Substrate	cpm	% P^{32} recovery	cpm	% P^{32} recovery
1	dAPPP + $TP^{32}PP$	—	—	20,000	100
2	$dAP^{32}PP$ + TPPP	19,500	100	—	—

FIG. 2.—Ultracentrifuge pattern of poly (dA + T) primed with poly (A + U). Pictures were taken at 2-min intervals at a speed of 59,780 rpm. The dark region represents the ultraviolet light absorbing solution. The $O.D._{260}$ of the poly (dA + T) was 0.4 in 0.2 *M* NaCl. The sedimentation constant is 28 S.

Equilibration was carried out at 8° but the value given is corrected to 25°. Equilibrations carried out at 25° showed considerable degradation of the poly (dA + T).

(b) *Light scattering:* Figure 3 shows the light scattering results for poly (dA + T). The weight-average molecular weight and radius of gyration are 1.8×10^6 and 2200 Å, respectively. The high radius of gyration is similar to that found for many natural native DNA's, indicating that the poly (dA + T) is a stiff chain. Its sedimentation constant is 21 S.

(c) *Helix-coil transitions:* Further evidence for the existence of an ordered structure in this polymer is given in Figure 4, where the change in absorption at 260 mμ is plotted as a function of temperature. The hyperchromic shift is quite sharp and occurs at 58° in 0.1 *M* NaCl and 41.2°C in 0.01 *M* NaCl. On fast cooling the optical density returns to its original value, indicating a reversible helix-coil transition. The values for the melting point at different ionic strengths are similar to those reported for the alternating poly dAT copolymer.[21] Figure 5 shows melting curves for two reaction mixtures in which there was approximately a one-fold synthesis. It is apparent that only two transitions are present: one for the product poly (dA + T) and one for the primer poly (A + U).[22]

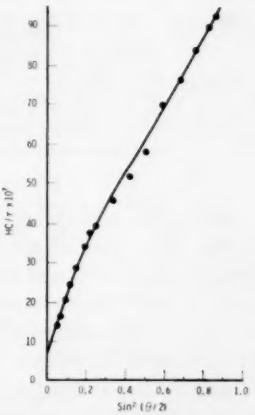

FIG. 3.—Light scattering results of poly (dA + T) primed with poly (A + U). The curve represents the zero concentration line of a Zimm plot. The ordinate intercept is the reciprocal of the weight-average molecular weight. The limiting low angle slope yields the radius of gyration. Molecular weight, 1.8×10^6; radius of gyration, 2200 Å.

(4) *Chemical and structural requirements of the primer:* Neither poly A nor poly U alone was able to act as a template using dATP and/or TTP as substrates. The three-stranded poly (A + 2U) also gave no product with these substrates. Apparently a two-stranded helical template structure is required. Using poly (A + U as primer with either dATP or TTP alone did not result in polymer synthesis; hence both strands must be

synthesized *simultaneously* in order to produce a polymer.

As might be expected, we have also found that poly (C + I) primes a reaction with dGTP and dCTP as substrates. Further studies on the product are under way. If poly (C + I) acts as a template, the product should be poly (dG + dC), in which each chain is a homopolymer, identical to the product obtained in the unprimed reaction.[10] Poly I does not serve as a primer with dCTP as the substrate. Neither poly (A + I) nor poly (A + 2I) serve as primers. For the former, the product would be poly (T + dG) if a helical structure were possible. The lack of product may therefore be a reflection of the fact that T and dG cannot couple in a helical structure. This would also suggest that the two strands are not synthesized independently.

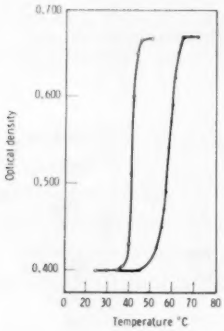

FIG. 4.—Optical density-temperature transition curves for poly (dA + T). Curve on left is in 0.01 *M* NaCl, melting point 41.2°; curve on right is in 0.1 *M* NaCl, melting point 58°. Measurements were made at 260 mμ.

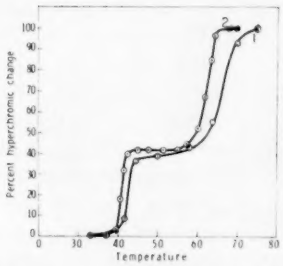

FIG. 5.—Hyperchromic change obtained on heating the reaction mixture after onefold, primed synthesis of poly (dA + T). The solvent is 0.02 *M* NaCl. One per cent duponol was also present, in curve 1. Measurements were made at 260 mμ.

Discussion and Conclusions.—It is evident that poly (A + U) serves as a primer in the DNA polymerase reaction, in that the lag period is greatly reduced. The fact that each chain of the product, poly (dA + T), is homopolymeric shows that poly (A + U) is the template. The poly (dA + T) product has a high molecular weight and an ordered structure. Since the melting curve of the reaction mixture of the onefold synthesis shows transitions only for the product and the template, it may be concluded that no hybrid intermediate is formed in measurable amount. Such a hybrid would presumably consist of a poly A strand complexed with the newly formed poly T strand; likewise, the poly U would be complexed with the poly dA. This would imply strand separation during synthesis. However, if the reaction were to continue, the hybrid molecules would then have to unwind so that the original poly A and poly U strands could reunite to form the helical poly (A + U); this would be a stringent requirement since we have shown that neither single-strand poly A nor poly U acts as primer in this reaction. The presence of most, if not all, of the original poly (A + U) after onefold synthesis is shown by its melting curve. Alternatively, one might postulate that only a

minute fraction is functional and that the hybrid it forms can itself act as template; but this appears unlikely since the system is always saturated with polymerase. It is thus unlikely that strand separation is involved in the function of poly (A + U) as a template.

The DNA and RNA polymerase seem to function in an almost identical manner; both utilize nucleoside triphosphates as substrates; both can bring about the incorporation of ribonucleoside triphosphates, in the presence of Mn^{++}.[11] Both reactions are primed by DNA and yield RNA or DNA products having the base composition[12–14] and nearest-neighbor pattern[8, 15] of the primer. The products, both RNA and DNA, are complementary and antiparallel to their templates, and both strands of the DNA can prime either product.[1, 16, 17] Since RNA polymerase utilizes helical DNA[2, 5] and poly (A + U)[18, 19] as templates, leaving them intact, and since we have shown the same is true of helical poly (A + U) in the DNA polymerase system, it is reasonable to conclude that with the DNA polymerase the conserved unit of DNA is the double helix.[3]

There is an interesting difference between the DNA and RNA polymerase systems when poly (A + U) is used as primer. With DNA polymerase our data show that dA and T are incorporated into the product to the same extent, whereas Nakamoto and Weiss[18] and Krakow and Ochoa[20] have shown that the amount of UTP incorporated exceeds that of ATP in the RNA polymerase system. It is possible that this type of difference provides a basis for the synthesis of noncomplementary RNA *in vivo*—there may be a preferential interaction of RNA polymerase with just one of the DNA chains at certain loci.

Evidence that certain other helical double-stranded, but not single- or triple-stranded, polyribonucleotides can prime the DNA polymerase reaction has also been presented.

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*POLYPLOIDY IN THE MEXICAN AXOLOTL (AMBYSTOMA MEXICANUM) RESULTING FROM MULTINUCLEATE OVA**

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Spontaneous polyploidy in the Amphibia has been reported by several investigators.[1–4] Such polyploids among the progeny of diploid parents generally have been interpreted as resulting from one or more of the theoretically possible accidents which may occur in meiosis, fertilization, or the first cleavage division. This interpretation is much in accord with the fact that polyploidy may be induced by experimental procedures which suppress the second maturation division or disturb the normal progress of the first cleavage division, and it doubtless accounts for the great majority of spontaneous polyploids. Investigators rarely have offered the interpretation that polyploidy may result from a multinucleate condition in the egg. Humphries,[5] for example, hesitated to assume that diploidy in the egg of Triturus might be the result of a binucleate condition, in view of "the direct pathways actually seen to exist." More recently, however, Parmenter *et al.*[6] have reported the occurrence of binucleate and trinucleate young oöcytes in *Rana pipiens*. Their discovery of this condition suggested, as they point out, an important possible source of diploid parthenogenetic individuals. They point out, too, the possibility that the failure of one of the nuclei in a binucleate egg to undergo one or both meiotic divisions "could produce various chromosome numbers in mature eggs and in embryos resulting from their parthenogenetic stimulation or from fertilization."

Whether the multinucleate young oöcytes of *R. pipiens* observed by Parmenter *et al.* would have given rise to mature ova of unusually large size is uncertain. Briggs[7] states that he has observed eggs from females of this species with two or even three first polar bodies, but that they were of essentially the same size as mononucleate eggs, and could not be distinguished without the aid of a microscope. Multinucleate eggs of the axolotl, however, are readily distinguished by their larger size when eggs of a spawning including them are examined with the naked eye (Fig. 1). Their markedly large size, when they were first observed by the writer, caused them to be recorded as "giant" eggs.

The first oversized, or "giant," egg was found in 1953 in a spawning from a homozygous dark (D/D) female. No others were observed until 1956 when several appeared in two successive spawnings of a second female which was closely related to

the first (the daughter of a sister). All of these eggs happened to be fertile and were in cleavage stages when first observed. Later, when similar large eggs began to appear with considerable frequency in the spawnings of descendants of this second female, some were observed before cleavage began. Such eggs were usually found to have first polar bodies formed at two sites, commonly at some little distance apart (Fig. 1). If the egg was one which had been fertilized, second polar bodies later appeared at these sites. In a few spawnings, however, some of the oversized eggs were distinctly larger than the others, and in these, polar bodies were observed at three or even four sites instead of two. In one instance a giant egg was found with a first polar body formed at only one site instead of the two or more characteristic of such eggs. Unfortunately, this egg was not sectioned to determine whether a second nucleus was present in a submerged position, such as was described by Humphries[5] for the nucleus of occasional mononucleate eggs of Triturus.

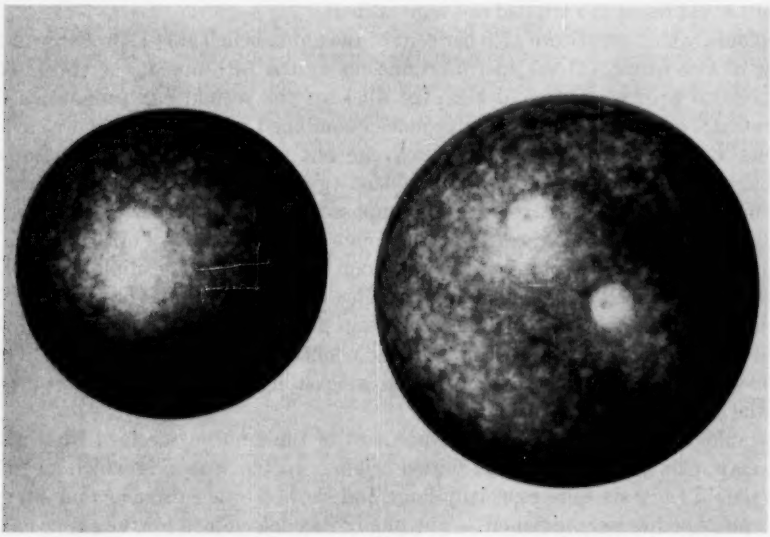

FIG. 1.—Mononucleate and binucleate eggs of the axolotl, fixed after formation of the first polar body. ×24.

Females which produced giant eggs in one spawning tended to do so in others. Percentages of such eggs, in the spawnings including them, ranged from 0.13 per cent (1 in 768) to 6.24 per cent (29 in 465). In a group of 51 spawnings for which complete records were available, 209 giant eggs were included in the total of 24,588, an incidence of 0.85 per cent. This rate is probably slightly below the actual one, since large eggs which were at all doubtful because of damage or cytolytic changes were disregarded.

Determinations of the ploidy of embryos or larvae developing from giant eggs were made whenever possible. This was generally done from tailtip preparations stained with azure B, in which the maximum number of nucleoli per cell can easily be ascertained. This number serves to indicate the number of sets of chromosomes present.[8] In a few embryos the number of nucleoli was found by examination of un-

TABLE 1

PLOIDY OF 145 INDIVIDUALS WHICH DEVELOPED FROM OVERSIZED OR GIANT EGGS, PRESUMABLY ALL MULTINUCLEATE

Type	Number found	Percentage of total
Diploid (*2N*)	26	17.9
Triploid (*3N*)	113	77.9
Tetraploid (*4N*)	4	2.8
Mosaics (*2N/4N* and *2N/3N + N*)	2	1.4

stained cells with a phase microscope. In all, the ploidy of 145 individuals was determined. These are included in Table 1.

The great majority of the embryos from giant eggs (77.9%) were found to be triploids. Since most such eggs showed two sites at which first and second polar bodies were formed, they would appear to be binucleate, and after completion of meiosis would contain two pronuclei, each with the haploid number of chromosomes. These, combining with the set of chromosomes introduced by the spermatozoon, would result in a triploid cleavage nucleus.

Diploids, which constitute 17.9 per cent of the animals in Table 1, probably arose in one of two ways: (1) the sperm pronucleus united with one of the two female pronuclei to produce a diploid cleavage nucleus, the second egg pronucleus degenerating; or (2) the two female pronuclei combined to form a diploid cleavage nucleus, the spermatozoon inducing cleavage but contributing no chromosomes. Although this second mode of development (gynogenesis) sometimes occurs in mononucleate eggs of the axolotl,[3] it is impossible to say whether it actually accounts for any of the diploid embryos from binucleate eggs. Proof of the participation of the chromosomes of the spermatozoon in producing the diploid cleavage nucleus was obtained in one instance in which a dark female (D/D) had been mated with a white male (d/d). One of her giant eggs produced a diploid larva which was reared and mated with a white animal; the resulting progeny numbered 67 darks and 59 whites, showing that this one diploid, at least, had received a chromosome set from the spermatozoon.

Tetraploids, which constituted 2.8 per cent of the embryos derived from giant eggs, may, like the diploids, be of varied origin. In the spawning which included 29 oversized eggs, six were especially large and showed either three or four sites at which polar bodies were given off. The ploidy was determined for the embryos developing from four of these eggs. Three were triploids and one was a tetraploid. The latter could have come from a trinucleate egg in which all three nuclei completed meiosis, the three pronuclei resulting then combining with the sperm pronucleus to produce a tetraploid cleavage nucleus. Another possibility is that this tetraploid was derived by gynogenesis from one of the eggs with four nuclei, all of which had completed meiosis. The three triploids derived from eggs with three or four nuclei could have arisen by various combinations of three haploid nuclei from the four or five present (sperm pronucleus, plus three or four egg pronuclei).

Whether the mosaics listed in Table 1 came from binucleate eggs or ones with three or four nuclei is uncertain. One possible origin for the *2N/4N* mosaic is that it came from an egg with three nuclei, one of which, after meiosis, combined with the sperm pronucleus to form a diploid cleavage nucleus; the other two egg pronuclei, if shifted into one of the first two blastomeres, could then unite with the diploid nucleus of that blastomere to produce the tetraploid condition found in one lateral

half of the mosaic. The reader may find other possible origins for this mosaic, and perhaps also an explanation for the one which was $2N/3N + N$.

Possible Genetic Basis for Tendency to Produce Multinucleate Ova.—The occurrence of binucleate eggs in any amphibian species must depend upon the occasional failure of completion of a final oögonial division. Nuclear division without formation of separate daughter cells, followed shortly by onset of meiosis in the two nuclei, would give rise to the binucleate young oöcytes described by Parmenter *et al.*[6] for *Rana pipiens*. Mitotic division of one of the two daughter nuclei before meiosis begins, again without cell division, would result in a trinucleate oöcyte, and if such mitosis occurred in both daughter nuclei, an oöcyte with four nuclei. Whether the tendency toward incomplete division in the final mitoses of the oögonia in frog and axolotl is inherent in the character of these cells themselves or results from some peculiarity of their environment (some unusual feature of ovarian structure, possibly) remains problematic. In either case, the phenomenon may very well have a genetic basis.

With minor exceptions which will be mentioned subsequently, all of the axolotl females known to have produced giant (multinucleate) eggs are descended through both parents from the second female which spawned such eggs. The dark strain to which these animals belong was derived from a few young animals obtained in 1951 from Dr. J. Holtfreter of the University of Rochester. These animals were descendants of axolotls imported by Dr. E. Caspari, then at Cold Spring Harbor; most of them had been procured for him by Dr. H. Gloor from various European dealers and were, as Gloor states,[9] " of an entirely obscure origin."

Figure 2 shows the earlier ancestry of the animals in the writer's colony which have produced giant eggs. The animals numbered 2, 3, and 4 were among those obtained from Holtfreter. From spawning no. 542, four females were reared, of which one (542-1) produced a single giant egg. For the 24 spawnings from four females of spawning no. 607, not a single unusual egg was recorded. The third-generation female no. 755-4, on the other hand, produced 16 giant eggs in three of her four spawnings. Only two of her daughters were reared (872-3, 872-4); these laid no oversized eggs in nine and eight spawnings, respectively. From matings with their brother, no. 872-2, however, they both produced daughters which spawned a few such eggs. The descendants of these three animals of spawning 872 now include 179 homozygous dark (D/D) females which have spawned one or more times, of which 69 (38.6%) have produced one or more giant eggs. The high percentage of females of this particular dark strain showing a tendency to lay multinucleate eggs is in striking contrast with the absence of such females in the white

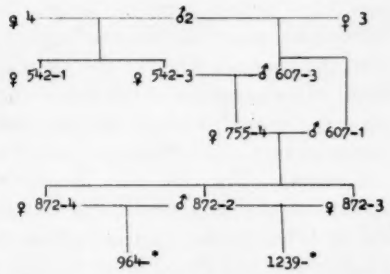

FIG. 2.—Chart showing matings of dark axolotls from which all females producing oversized (multinucleate) eggs are descended. The animals numbered 2, 3, and 4 were obtained from the Holtfreter colony at the University of Rochester. Female 542-1 was the first known to lay an unusually large egg. Female 755-4 laid several such eggs; all other females known to have spawned such eggs are her descendants by way of the three animals of spawning no. 872.

* Some of the many females from these spawnings produced multinucleate eggs.

strain and four other dark strains from which, all told, well over a thousand spawnings have been obtained.

The data on record would appear to support the interpretation that the tendency to produce multinucleate eggs depends upon homozygosity for a recessive gene. If we assume that this gene was carried by female no. 4 and male no. 2, it could have been transmitted through both parents to every homozygous dark (D/D) female of this strain which has since spawned multinucleate eggs. Although a few heterozygous dark (D/d) females have also produced such eggs, their white fathers could have carried the recessive gene for this peculiarity as well as their dark mothers, since two of these white males were actually descended from male no. 2, and the third had as one ancestor a dark male obtained from the Holtfreter colony at the same time as male no. 2 and assumed to be his sib. Ten heterozygous dark (D/d) females whose white (d/d) father was of an unrelated strain (inbred since 1937) produced not a single oversized egg in 20 spawnings (9,921 eggs) although their mother had laid such eggs and two of her five daughters by a dark male had likewise done so.

One rather exceptional finding may be mentioned. A dark female whose right ovary had been partially replaced by a graft from a white donor spawned a single giant egg in each of two spawnings. She was always mated with white males, and since the triploid larvae from her two giant eggs were white rather than dark (the dominant color), these eggs must have come from her ovarian graft rather than her own ovary. Both parents of the white embryo furnishing this ovarian graft were descended from the dark male already mentioned as a probable sib of male no. 2 of Figure 2, and it is possible, therefore, that this white donor was homozygous for the postulated recessive gene. Among the few siblings of this white embryo which were reared were four females, none of which ever spawned giant eggs. We are left with the question whether the giant eggs derived from the ovarian graft were the result of the genotype of the donor embryo or were induced by influences depending upon the constitution of the dark host, which, however, never laid giant (multinucleate) eggs coming from her own ovary.

Comment.—The discovery of multinucleate eggs in the spawnings of Mexican axolotls of this dark strain, and the fact that a high percentage of these eggs give rise to heteroploids, does not affect the validity of the interpretations offered by Fankhauser and Humphrey[3] for the heteroploids they reported in this species. Those heteroploids, with the exception of nine at most, were the progeny of white axolotls or of animals of dark strains for which multinucleate eggs were never observed. Their heteroploidy, therefore, must have resulted from the various accidents of meiosis, fertilization, or the first cleavage division which were postulated by the authors. Substantial evidence for the stated origins of many of those heteroploids was afforded by their color or sex, or their genotypes as determined by test matings.

That multinucleate eggs do occur in the Mexican axolotl, however, must be borne in mind by investigators undertaking studies involving spontaneous or experimental heteroploidy in this species. Descendants of the dark axolotls "of obscure origin" sent by Gloor to Cold Spring Harbor are now a part of many other laboratory colonies. The dark stock in the writer's own colony at Indiana University for the past several years has consisted exclusively of the descendants of the animals of this

strain included in Figure 2, and eggs or larvae of this stock have been furnished to several investigators desiring to establish colonies of their own. Fortunately, the multinucleate eggs of these axolotls are easily recognizable because of their large size and hence can be excluded from investigations in which their character might lead to erroneous conclusions. Their occurrence and easy recognition are, of course, advantageous to an investigator who has use for the polyploids to which they commonly give origin.

Summary.—Multinucleate eggs, much larger than the ordinary mononucleate ones, have been found in the spawnings of dark Mexican axolotls of a strain derived from European stock of obscure origin. The majority of these oversized eggs have two nuclei, but a few have three or four. In 51 spawnings including them, such large eggs constituted 0.85 per cent of the total (209 out of 24,588). Of 145 embryos or larvae which developed from these eggs, 113 (77.9%) were triploids, 26 (17.9%) were diploids, and the remaining 6 were tetraploids and mosaics.

The females spawning multinucleate eggs, with one exception, have been descended through both parents from the two animals whose mating produced the first female known to have spawned such eggs. This suggests that the tendency to produce multinucleate eggs may depend upon homozygosity for a recessive gene or genes. Such eggs have not been found in the spawnings of white axolotls or of dark females of four other strains, but have been included in spawnings of over 38 per cent of the females of the dark strain showing this peculiarity. Their occurrence in this strain must be taken into account in any study involving consideration of chromosome number.

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*EQUIVALENCE OF HYDROGEN AND THIOSULFATE IN BACTERIAL PHOTOSYNTHESES**

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Photosynthesis of plants is currently interpreted as a process in which absorbed radiant energy is partly converted into "assimilatory power" which, in the form of ATP[1] and PNH_2, is required for the synthesis of organic matter from CO_2. ATP can be formed by both cyclic and noncyclic photophosphorylation; only in the latter case is ATP production coupled with PN reduction, through an enzyme-mediated transfer of electrons from an excited pigment complex and H ions from the medium. The "holes" in the pigment complex are filled by electrons derived from OH ions, with a concomitant evolution of O_2.

For bacterial photosyntheses, in which O_2 is not produced, the presence of an exogenous oxidizable substrate is required. If the potential of the substrate is such that it can serve for a direct enzymatic PN reduction, one may argue that "the contribution of light . . . is . . . limited to the formation of ATP by cyclic photophosphorylation";[2] this applies, for example, to H_2. But if the substrate cannot be so used, a noncyclic electron flow must be invoked in which the H- or electron donor, e.g., thiosulfate, supplies electrons directly to the oxidized pigment complex.

Cyclic photophosphorylation has been demonstrated with intact or fragmented chloroplasts[3] and with bacterial chromatophores;[4] but its occurrence in intact cells has not been unequivocally established. It is true that such a process would suffice to account for the light-dependent synthesis of succinate from CO_2 and propionate by *Chlorobium thiosulfatophilum*;[5] it has also been used to explain the photoassimilation of acetate to poly-β-OH butyrate by *Rhodospirillum rubrum* and *Chromatium* spec., and its enhancement by the simultaneous presence of H_2.[6–8] Cyclic photophosphorylation would supply ATP for substrate activation, while PNH_2, needed for the reduction of acetoacetate, would be formed exclusively by light-independent reactions, particularly from PN and H_2. Without denying the plausibility of this interpretation, we do not believe that the experimental results preclude the possibility of PNH_2 formation in a noncyclic photochemical process.

Moreover, the light-dependent syntheses of succinate and poly-β-OH butyrate bear little resemblance to normal photosynthesis in which CO_2 is the sole carbon source for the manufacture of organic cell constituents. And the assumption that also in the latter case different substrates cause PN reduction by different mechanisms seems at variance with the identical quantum numbers for CO_2 assimilation, by both *Chromatium*[9] and *Chlorobium*,[10] with either H_2 or thiosulfate, despite the large differences in energy requirements. This very fact favors the concept of a greater similarity in the photosynthetic mechanisms.[5]

Nevertheless, Arnon *et al.*[8] have rightly claimed that the quantum efficiency argument is valid only if, under the conditions used for determining the quantum numbers, the products of CO_2 assimilation with H_2 and thiosulfate were qualitatively and quantitatively similar, since the ATP requirements for various biosynthetic proc-

esses differ considerably; and it has been shown that in *Chromatium* the products of photosynthesis can be greatly modified by the addition of certain oxidizable substrates.

Hence it was important to compare the pattern of CO_2 assimilation by *Chlorobium* in the presence of H_2 and thiosulfate, and the results of our experiments are here reported.

Methodology.—Cultivation of organisms: *Chl. thiosulfatophilum* (strain Larsen) was grown in Pfennig's[11] medium, supplemented with 0.05% NH_4-acetate, 1% NaCl, and 0.1% $Na_2S_2O_3 \cdot 5H_2O$, at 22–25°C, in screw-capped bottles, incubated alternately in light (16 hr; 40-watt incandescent bulbs at 40 cm distance) and darkness (8 hr).

Manometry: A conventional Warburg apparatus, identical with Larsen's,[12] was used; experimental details are given in the legends to the tables.

Illumination: Measurements of CO_2 assimilation rates at different light intensities served to determine the conditions for conducting experiments above or below saturation. Light intensities were varied by using banks of incandescent bulbs of different wattage, or by changing the distance between the light source and manometer flasks.

Gas atmospheres: N_2,[13] rendered O_2-free by passage over heated Cu, or H_2; the flask-manometer assemblies were flushed with the appropriate gas for 15 min.

Fractionation: The contents of a manometric flask were transferred to a chilled test tube, along with an equal volume of a freshly prepared, chilled cell suspension with which the flask was washed. After addition of TCA to a final concentration of 10%, the contents were fractionated by the procedure of Roberts *et al.*,[14] as modified by Arnon *et al.*,[8] which permits the separate extraction of poly-β-OH butyrate. However, complete separation of the supernatant fluid from the cell mass following centrifugation of the $CHCl_3$-extracted cell suspension proved impossible; the pellet tended to redisperse. Hence, only a small aliquot of the extract was removed for determining the radioactivity of the polymer. The remaining pellet plus supernatant fluid was then evaporated to dryness, and the residue treated with a warm, aqueous 0.05% detergent ("Hemosol") solution. This yielded a finely dispersed suspension, aliquots of which gave satisfactorily reproducible counts. The radioactivity of the "residual protein" fraction was computed by subtracting from the total counts the contribution of poly-β-OH butyrate.

In view of the general agreement as to the composition of the individual fractions, we have not chemically analyzed them; they are listed in the tables in conformity with the practice of Arnon *et al.*[8] Radioactivity was measured by conventional methods, with a thin-window GM tube.

Results —CO_2 assimilation by Chlorobium: At a light intensity slightly below saturation (80%), the assimilation patterns with either H_2 or thiosulfate are essentially similar: about 90 per cent of the incorporated C^{14} is found in the metabolic intermediate and protein fractions (Table 1). Doubling the exposure time leads

TABLE 1

DISTRIBUTION OF C^{14} IN ASSIMILATION PRODUCTS OF $C^{14}O_2$ BY *Chlorobium thiosulfatophilum* IN THE PRESENCE OF H_2 OR THIOSULFATE*

Period of illumination:	1 hr		2 hr	
Electron donor:	$S_2O_3^=$	H_2	$S_2O_3^=$	H_2
Fraction				
Metabolic intermediates	32.8	65	26.8	44.5
Polysaccharides and nucleic acids	3.6	2.8	8.3	5.9
Poly-β-OH butyrate	1.1	1.0	0.72	1.1
Protein, total	62.5	31.3	65	49.5
Alcohol soluble	23.6	9.4	24	15.4
Residual	39.0	21.9	41	33.9
Per cent C^{14} recovery	108.5	98	108	100
Total C^{14} incorporated (cpm)	5.5×10^4	3.2×10^4	1.5×10^5	8.5×10^4

* Warburg flasks, *ca.* 16 ml capacity; main compartment: 2.2 ml Pfennig's medium without CO_2 and $Na_2S_2O_3$, but with 1% NaCl and either 0.05 *M* (thiosulfate exp'ts) or 0.01 *M* (H_2 exp'ts) phosphate buffer, pH 6.7; 1.8 mg (dry wt.) cells. Side arms: 4 μM $Na_2S_2O_3$ and 10 μM $Na_2C^{14}O_3$, respectively; gas phase N_2; or dilute HCl and 10 μM $Na_2C^{14}O_3$, respectively; gas phase H_2. Light intensity 80% of saturation. Numbers represent % of total incorporated C^{14} in fractions.

to relatively more label being fixed in the protein, and less in the intermediate metabolic products.

Virtually identical results were obtained in experiments where the light intensity was clearly above, or only 30 per cent of saturation, and when both H_2 and thiosulfate were present simultaneously.

Ordinarily the CO_2 fixation rates in the presence of H_2 or thiosulfate were the same, as already noted by Larsen.[12] But in some of our experiments the rate with thiosulfate was greater. We attribute the discrepancy to the fact that in the H_2 runs the pH was not accurately controlled. Here the phosphate concentration of the cell suspension was reduced from 0.05 to 0.01 *M* in order to eliminate the inhibitory effect of higher concentrations on CO_2 assimilation in the presence of H_2.[12] Owing to the low buffer capacity, the subsequent addition of 10 μM of $Na_2C^{14}O_3$ caused a marked rise in pH; and although this was offset by simultaneously adding dilute HCl from the second side arm, it was not possible to avoid differences in the final pH of various cultures.

With a view to duplicating as nearly as possible the conditions Larsen[12] used for his quantum determinations, most of our experiments were performed with thin cell suspensions (1.8 mg dry wt. cells per flask). Even so, as could have been expected from the results obtained at different light intensities, the assimilation patterns obtained with fivefold denser cell suspensions (10 mg dry wt. per flask) were no different from the others, regardless of light intensity.

Acetate assimilation by Chlorobium: When C-1 labeled acetate instead of CO_2 was used as the sole carbon source, the distribution of C^{14} in the fractions was essentially the same as in the experiments discussed previously, about 90 per cent of the label being recovered in the metabolic intermediate and protein fractions, again independent of the nature of additional substrates, and at both limiting and saturating light intensities (Table 2 *A*). At light saturation a small but significant fraction of the label was found in the $CHCl_3$ extracts (poly-β-OH butyrate), and about twice as much when either H_2 or thiosulfate was also present.

A similar labeling pattern was observed when both labeled acetate and unlabeled CO_2 were provided simultaneously (Table 2 *B*). The bulk of the label was once more found in the metabolic intermediate and protein fractions, but a larger proportion than usually, *ca.* 20–30 per cent, now appeared as polysaccharide. This is in line with Sadler and Stanier's finding that CO_2 is required for polysaccharide synthesis from acetate by *Chlorobium*.[15] The amount of label in lipids was negligible.

The data on total C^{14} incorporation, normalized to equal exposure times, show that in the absence of inorganic reductants CO_2 affects the rate of acetate assimiliation but slightly, though it markedly increases polysaccharide formation. In the absence of CO_2, both H_2 and thiosulfate strongly depress the assimilation, while in its presence both reductants cause a tenfold increase.

Discussion.—The essentially similar pattern of CO_2 assimilation by *Chlorobium* in the presence of either H_2 or thiosulfate implies that the energy (ATP) requirements for biosynthetic functions must be closely comparable in the two cases. Nonetheless, Larsen[10, 12] has shown that under conditions like those used in our experiments, the quantum numbers are identical. We believe that this is incompatible with the assumption[2, 7] of fundamentally different mechanisms for the

generation of "assimilatory power" (PNH_2 and ATP) with the two reductants.

In chemosynthetic bacteria where CO_2 assimilation involves only dark reactions, such differences seem to exist, for here the nature of the oxidizable substrate does influence the amount of CO_2 assimilated. In this context the following comparison is instructive: *Hydrogenomonas* spp., growing at the expense of H_2 oxidation, use only $^1/_4$ to $^1/_5$ as much O_2 for the assimilation of a given amount of CO_2[16] as do *Thiobacillus* spp., growing at the expense of thiosulfate.[17] Since, per mole of O_2 consumed, more energy is released in the oxidation of H_2 than of thiosulfate, this implies that the greater chemical potential of the former substrate contributes to the generation of "assimilatory power." This can best be explained by invoking a direct enzymatic reduction of PN with part of the H_2, the remainder being used for ATP formation by oxidative phosphorylation. Thiosulfate cannot serve as a direct reductant of PN, so that the thiobacilli must form PNH_2 by a different, currently obscure mechanism.

Now, the distinguishing feature of photosynthetic bacteria is that they have evolved a special organelle, the chromatophore system, for the conversion of radiant energy into chemical potential. Its structure closely resembles that of green plant chloroplasts, where PNH_2 is formed during a noncyclic transport of electrons from an excited pigment complex with which a small number of specific electron acceptor and donor molecules are intimately associated.[18] That the chromatophore system can function in a fully comparable manner follows from the fact that substrates such as thiosulfate can be used in bacterial photosynthesis. This shows that the organisms contain the necessary machinery for operating a noncyclic mechanism. And there is no *a priori* reason for assuming that this mechanism does not function in the oxidation of substrates such as H_2. As shown in the diagram, the essential

TABLE 2

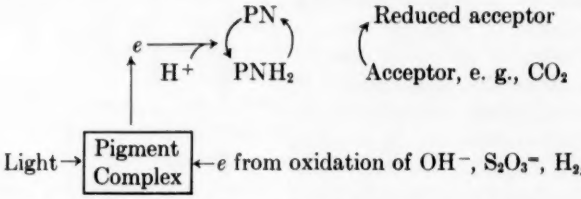
PHOTOASSIMILATION OF ACETATE-1-C^{14} BY *Chlorobium thiosulfatophilum* IN THE PRESENCE OF H_2 OR THIOSULFATE*

Added substrate:	*A*: CO_2 Absent, Light Intensity—				*B*: CO_2 Present, Light Intensity—			
	80% of Saturation—		Saturating—		80% of Saturation—		Saturating—	
Fraction	None	$S_2O_3^=$	None	$S_2O_3^=$	None	$S_2O_3^=$	None	$S_2O_3^=$
Metabolic intermediates	22.2	33	26.6	31.9	26	19.2	11.4	12.6
Polysaccharides and nucleic acids	8.6	10	6.9	5.1	13	22.5	15	22
Poly-β-OH butyrate	1.3	0.4	2.8	4.5	0.9	0.3	1.6	1.2
Protein, total	67.5	55.5	63.7	58.5	58.1	56.8	71.9	64.3
Alcohol soluble	31.5	23.5	28.2	28	28.1	30.3	22.4	31
Residual	36	32	35.5	30.5	30	26.5	49.5	33.3
Per cent C^{14} recovery	119	127	95.5	106	114	123	105	117
C^{14} incorporated per 100 min (cpm × 10^{-6})	2.5	1.5	3.6	2.0	3.4	7.0	4.5	16.0

Added substrate:	*A*: CO_2 Absent, Light Intensity—		*B*: CO_2 Present, Light Intensity—	
	80% of Saturation—		80% of Saturation—	
Fraction	H_2	H_2	H_2	H_2
Metabolic intermediates	18	19.3	15	18.4
Polysaccharides and nucleic acids	9.9	11.1	30	31.1
Poly-β-OH butyrate	0.5	5.0	0.9	0.9
Protein, total	70.5	64.8	52.8	49.7
Alcohol soluble	39.5	38	32.6	26.4
Residual	31	26.8	20.2	23.3
Per cent C^{14} recovery	105	99	113.5	104
C^{14} incorporated per 100 min (cpm × 10^{-6})	1.7	1.4	6.5	10.0

* Conditions as for Table 1, except that Na-acetate-1-C^{14} (7 μ*M*, 5μC/μM) was substituted for $Na_2C^{14}O_3$; where indicated, 10 μM unlabeled Na_2CO_3 added as well.

feature of a noncyclic mechanism is that the rate of PNH_2 formation, and hence of CO_2 assimilation, strictly depends on photochemical events, while the substrate supplies electrons to the electron-deficient pigment complex:



As will now be shown, such a mechanism can logically account for a number of facts that are otherwise difficult to explain.

It is currently envisaged that the electrons for PN reduction are raised to the requisite level in two one-quantum reactions.[18, 21, 22] Hence, the number of quanta needed for the assimilation of a CO_2 molecule would be 8, regardless of the nature of the oxidizable substrate; this is precisely what has been found.[9, 10, 12]

The substrate oxidation provides an obvious mechanism for the generation of ATP. But its yield per electron transferred may vary with the nature of the substrate, and be greater in H_2 than in thiosulfate oxidation. In that event the amount of ATP formed concomitantly with a unit quantity of PNH_2 would also be greater in the former case Now, since our results show essentially similar ATP requirements for CO_2 assimilation with either substrate, this should imply that, during photosynthesis with H_2, ATP accumulates, and this, in turn, might lower the oxidation rate. The differential effects of moderate phosphate concentrations, which inhibit *Chlorobium* photosynthesis with H_2 but not with thiosulfate,[12] could then be attributed to the establishment of different internal ATP/ADP ratios in the two cases.

The noncyclic electron flow mechanism also provides a tentative explanation for the curious fact that, in the absence of CO_2, acetate assimilation is inhibited by H_2 and thiosulfate, while it is greatly enhanced by these reductants in the presence of CO_2. The pattern of labeling with C^{14}, derived from either CO_2 or the carboxyl group of acetate, is so similar that we may conclude that acetate is partly oxidized to CO_2. The rate of this oxidation is determined by that of PNH_2 formation, which in turn depends on the rate at which PN is regenerated. Thus, in the absence of exogenous CO_2 the rate-limiting process will be the formation of CO_2 from acetate. Because acetate, H_2, and thiosulfate are oxidized by different enzymes, these substrates can be used simultaneously for the reduction of the electron-deficient pigment system. Hence, the rate of acetate oxidation and assimilation would be diminished in the presence of H_2 and thiosulfate.

Conversely, the availability of exogenous CO_2 can cause a maximum rate of PN regeneration, which would also permit an increased electron flow from the substrates, including acetate. In the absence of H_2 or thiosulfate, CO_2 affects the assimilation of acetate but little. This indicates that acetate is oxidized very slowly, a contention which is supported by the fact that *Chlorobium* cannot be grown at the expense of light, CO_2, and acetate. Addition of H_2 or thiosulfate increases the rate of PNH_2 formation and hence of CO_2 assimilation, which might entail the concomitant

assimilation of acetate. This is in line with the observation of Sadler and Stanier[15] that the growth of *Chlorobium* in a mineral H_2S or thiosulfate medium is more abundant if the medium is supplemented with acetate, and that this effect of acetate on cell yield is restricted to the period during which the inorganic reductant is present.

Our data show that, in contrast to *Rhodospirillum*[6] and *Chromatium*,[8] *Chlorobium* produces very little poly-β-OH butyrate from acetate, though in the absence of CO_2 the amount is twice as large if either H_2 or thiosulfate is also present. The identical effects of these two substrates again suggest that a fundamentally similar mechanism is involved in their use as reductants. But the small amounts of polymer recovered made us wonder whether our analytical procedure could have been responsible for the low values. Hence, we have also conducted some preliminary experiments on poly-β-OH butyrate formation from C-1-labeled acetate by *Chromatium* strain D. Here the yields were much greater, 20 per cent of the incorporated label being recovered in the polymer fraction if either H_2 or thiosulfate was added along with the acetate, a result which further emphasizes the identical effects of the two reductants.

This equivalence of H_2 and thiosulfate, as shown by the identical quantum numbers as well as by their effects on acetate assimilation and poly-β-OH butyrate formation, is a logical consequence of the concept that the chromatophore structure enforces a noncyclic electron flow in bacterial photosyntheses with various H- or electron donors. The assumption that some substrates serve as primary reductants of PN, in which case the absorbed radiant energy causes a cyclic photophosphorylation, fails to account for these facts. This raises the question whether there is compelling evidence for the occurrence of a cyclic process. The synthesis of poly-β-OH butyrate from acetate has been so interpreted.[6-8] But in both *Chlorobium* and *Chromatium* this process is enhanced equally by H_2, potentially a direct PN reductant, and by thiosulfate, which cannot serve in this way. In the latter case the PNH_2 needed for the reduction of acetoacetate must therefore be produced by a noncyclic mechanism, and a similar means of its formation from H_2 cannot be excluded.[19]

However, the polymerization of β-OH butyrate by Athiorhodaceae is not an oxidation-reduction reaction. In this respect it resembles the light-dependent synthesis of succinate from propionate and CO_2 by *Chlorobium*.[12, 20] Hence, these processes do not require the participation of PNH_2, but only of ATP for substrate activation.

The latter is somewhat simpler to analyze because *Chlorobium* can oxidize neither propionate nor succinate,[12, 20] which precludes a noncyclic electron flow. Thus, photochemically induced changes in the distribution of electrons in the pigment complex must be reversed by an internal migration of the affected electrons themselves. That this can be coupled with the formation of ATP is not in question. But the occurrence of such a cyclic photophosphorylation during this abnormal "photosynthesis" would then be the logical consequence of conditions which prevent the operation of a noncyclic mechanism. This argument also applies to the polymerization of β-OH butyrate, but here the situation is complicated by the fact that the polymer is a storage product which can further be used for cellular syntheses in the presence of CO_2 and light.

When first discovered,[3, 4] cyclic photophosphorylation was considered essential as a supplementary source of ATP, since the amount produced in a noncyclic process was deemed insufficient to meet the demands of CO_2 assimilation.[3] However, present information conflicts with this contention.

As stated earlier, it is currently held that in plants 2 quanta are involved in the photochemical events that yield an electron at a potential suitable for the reduction of PN.[18] Hence, 8 quanta are needed to produce the amount of PNH_2 required for the assimilation of a molecule of CO_2 to the carbohydrate level. But it has repeatedly been found that normal photosynthesis can be accomplished with no more than 8 quanta per CO_2 molecule. This leaves no radiant energy available for cyclic photophosphorylation, so that we are forced to conclude that the ATP requirement of plant photosynthesis can be fully met by noncyclic processes. That the same quantum number has also been found for normal bacterial photosyntheses strongly suggests that here, too, only a noncyclic electron flow ordinarily operates. Even though they occur in the infrared region, i.e., with quanta of lower energy content, this is counterbalanced by the need for an additional energy source in the form of an oxidizable substrate. The requisite amount of ATP can thus be produced in a noncyclic process, particularly because energy is not spent for the formation of O_2 from H_2O.

We conclude that the observed equivalence of H_2 and thiosulfate in *Chlorobium* photosynthesis favors the interpretation of all photosyntheses as primarily noncyclic mechanisms, imposed by the specialized photosynthetic organelles.

Summary.—Determination of the distribution of C^{14} among the assimilation products formed by *Chlorobium thiosulfatophilum* from $C^{14}O_2$ or $CH_3C^{14}OOH$ in the presence of either H_2 or thiosulfate has shown a fundamental similarity. This points to the primary involvement of noncyclic mechanisms in all photosynthetic processes.

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[1] Abbreviations: ATP, adenosine triphosphate; PN, PNH_2, oxidized and reduced forms of pyridine nucleotide, respectively; TCA, trichloroacetic acid.

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*CELL-FREE PEPTIDE SYNTHESIS DEPENDENT UPON SYNTHETIC OLIGODEOXYNUCLEOTIDES**

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Since the use of randomly ordered template RNA preparations to direct cell-free protein synthesis has provided a relatively simple experimental approach for investigating the nature of the genetic code,[1, 2] it seemed that chemically defined DNA preparations also might facilitate such investigations. There have been several reports of cell-free amino acid incorporation dependent upon natural DNA;[3–8] however, the limited number of defined DNA preparations, such as poly d(AT), poly dC, and poly dG, which can be synthesized with DNA polymerase,[9, 10] have hindered investigations in this area. The chemical synthesis of oligodeoxynucleotides containing up to approximately 15 bases per chain by the dicyclohexylcarbodiimide method of Khorana and his associates,[11, 12] and the demonstration of an oligodeoxynucleotide-dependent synthesis of polyribonucleotides, catalyzed by RNA polymerase,[13–18] afforded an opportunity to study the ability of such oligodeoxynucleotides to stimulate cell-free amino acid incorporation. Since poly A serves as a template for polylysine synthesis,[19] we have used synthetic oligodeoxythymidylate to direct poly A, and then polylysine synthesis, as shown below:

$$\text{ATP} \xrightarrow[\text{RNA polymerase}]{\text{Oligo dT}} \text{Poly A} + \text{PP} \qquad (1)$$

$$\text{Lysine} \xrightarrow[\textit{E. coli} \text{ extracts, etc.}]{\text{Poly A}} \text{Polylysine} \qquad (2)$$

The characteristics of the poly A and polylysine synthesis directed by chemically synthesized oligodeoxynucleotides, natural DNA, and poly U are described below.

Materials and Methods.—Synthesis of oligodeoxynucleotides: Nucleoside mono- and triphos-

phates were obtained from Pabst Laboratories, Milwaukee, Wis., and Calif. Corp. Biochem. Research, Los Angeles, Calif., and the purity of each was checked before use by paper chromatography employing solvents A, B, and C (see below). Oligonucleotides of deoxythymidine-5′ phosphate and deoxyadenosine-5′ phosphate were synthesized by the dicyclohexylcarbodiimide method, as described by Khorana and his co-workers, and were purified by column chromatography.[11, 12, 20] The chain length of oligo dT_{13-14} was determined by measuring the ratio of total phosphate[21] to terminal phosphate released after hydrolysis with *E. coli* alkaline phosphatase (Worthington Biochem. Corp., Freehold, N. J.).[22, 23] Other fractions were identified from their column and paper chromatographic mobilities.[12] Incubation with rattlesnake venom phosphodiesterase (Worthington Biochem. Corp.) converted oligo dT to deoxythymidine-5′ phosphate.[24] An ϵ at 267mμ, pH 7, of $9.6 \times 10^{-3}\ M^{-1}\ cm^{-1}$ was assumed for (pdT) residues in oligo dT when calculating (pdT) concentrations. Descending paper chromatography was performed with Whatman 40 and 3 MM paper for solvents A–D, and with Whatman DE-20 paper for solvent E. Solvent components were as follows: solvent A, n-propyl alcohol/concentrated NH_4OH/H_2O = 55/10/35 V/V; solvent B, isobutyric acid/1.0 *M* NH_4OH/0.1 *M* disodium ethylenediaminetetraacetate = 1,000/600/16 V/V; solvent C, isopropyl alcohol/concentrated NH_4OH/0.1 *M* boric acid = 7/1/2 V/V; solvent D, pyridine/n-butyl alcohol/acetic acid/water = 6/9/3/7 V/V; solvent E, 0.3 *M* ammonium formate.

Synthesis of polyadenylic acid (stage I): Tetralithium 8-C^{14}-ATP was obtained from Schwarz BioResearch, Inc., Orangeburg, N. Y. (0.15–1.5 mcuries/mmole). Calf thymus DNA was purified according to the method of Kay *et al.*[25] Poly A, poly U, and poly C, prepared with polynucleotide phosphorylase, were obtained from Miles Chem. Co., Clifton, N. J. *E. coli* RNA polymerase was purified 100- to 150-fold by the procedure of Chamberlain and Berg.[16] The final specific activity after the DEAE column step was 800–1600 units[16]/mg protein when assayed with calf thymus DNA. Each RNA polymerase stage I reaction mixture contained the following in a total volume of 0.125 ml; 0.04 *M* Tris pH 7.8; 4×10^{-3} *M* $MgCl_2$; 1×10^{-3} *M* $MnCl_2$; 1.2×10^{-2} *M* mercaptoethanol; 1.6×10^{-3} *M* ATP; oligo- or polynucleotide where specified, and 20 μg RNA polymerase protein (~20 units[16]). Reaction mixtures were incubated for 15 min at 37° unless otherwise noted. Poly A synthesis was determined concomitantly in separate reaction mixtures containing C^{14}-ATP in place of C^{12}-ATP. After incubation at 37° for 15 min, reaction mixtures were chilled, and 2.0 ml of cold H_2O and 1.0 ml of 15% TCA were added to each tube. After standing for 10 min at 3°, the contents of each tube were filtered with suction through a Millipore filter (0.45-μ pore size, 25 mm diameter, Millipore Co., Bedford, Mass.) and washed with 25 ml of 5% TCA at 3°. Filters were glued to planchettes, dried, and radioactivity was determined in a thin-window, gas-flow counter (Nuclear-Chicago Corp., Des Plaines, Ill.) with a counting efficiency of 25%.

The average chain length of C^{14}-poly A synthesized with RNA polymerase in a stage I incubation was determined in the following manner. A reaction mixture was heated at 90–95° for 5 min and then was chilled in ice. Precipitated protein was removed by centrifugation, and the supernatant fluid was lyophilized. The residue was dissolved in water and chromatographed on Whatman DE-20 paper with solvent E for 2.5 hr to remove C^{14}-ATP from the C^{14}-poly A which remained at the origin.[18] The C^{14}-poly A was hydrolyzed in 0.3 *N* KOH for 20 hr at 37°, and the solution was adjusted to pH 10–11 with Dowex 50 (H^+). Unlabeled carrier adenosine, adenosine-3′(2′)-phosphate, and adenosine-3′(2′)-5′-diphosphate (the gift of Dr. Leon Heppel) were added, and the components of the solution were separated by chromatography on Whatman DE-20 paper in solvent E for 6 hr, and also on Whatman 40 paper in solvent A for 24 hr. The chromatograms were cut into 0.5-in. strips, placed in vials containing toluene-PPO-POPOP phosphor solution,[26] and the radioactivity of each sample was determined in a liquid scintillation counter (Packard Instrument Co., Inc., La Grange, Ill.).

Synthesis of C^{14}-polylysine (stage II): The sources and specific radioactivities of C^{14}-amino acids, and the preparation of DNase-treated, preincubated, cell-free, *E. coli* extracts used in stage II (protein synthesizing) reaction mixtures have been described previously.[1]

Stage II components were added to stage I reaction mixtures immediately after stage I tubes had been incubated at 37° for 15 min. Thus, both stage I and II reactions were performed consecutively in the same tube. Each combined stage I and II reaction mixture contained the following components in a total volume of 0.25 ml: 6×10^{-2} *M* Tris, pH 7.8; 2×10^{-3} *M* $MgCl_2$;

1.2×10^{-2} *M* magnesium acetate; 5×10^{-4} *M* $MnCl_2$; 1.2×10^{-2} *M* mercaptoethanol; 2.8×10^{-3} *M* ATP; 5×10^{-2} *M* KCl; 5×10^{-3} *M* PEP; 5 μg crystalline PEP kinase (Calif. Corp. Biochem. Research); 2×10^{-4} *M* each of 19 L-amino acids; 2×10^{-4} *M* C^{14}-L-lysine (Nuclear-Chicago Corp.) of specific radioactivity 4–8 mcuries/mmole; 20 μg RNA polymerase protein and 1.1 mg *E. coli* extract protein. Reaction mixtures were incubated at 37° for the times indicated, and C^{14}-lysine incorporated into protein was then precipitated and counted by the method of Gardner *et al.*[19] unless otherwise specified. All assays were performed in duplicate.

Characterization of the product synthesized from C^{14}-lysine: After 30 min of incubation at 37°, each stage II reaction mixture was deproteinized by the addition of 2 ml of cold 10% TCA solution. The supernatant solution was extracted three times with an equal volume of ether, concentrated *in vacuo*, and then the residue was cochromatographed with a partial tryptic digest of chemically synthesized C^{12}-polylysine (YEDA Research and Development Co., Ltd., Rehovoth, Israel) on Whatman 3 MM paper in solvent D for 56 hr (similar to the solvent described by Waley and Watson[27]). C^{14}-Lysine moves rapidly in this solvent and can be separated easily from polylysine of chain length greater than 11 which remains at the origin. The C^{14}-product together with unlabeled carrier polylysine was eluted from the origin with 0.1 *N* HCl, then dried and hydrolyzed for 12 hr in 6 *N* HCl at 110°. The residue from the dried reaction mixture was rechromatographed in the same manner. The radioactivity of each chromatogram was determined as described earlier. C^{14}-material eluted from the origin was characterized further by paper chromatography and was incubated at 37° for 9 hr with 20 μg of trypsin in a 0.25 ml reaction mixture containing 0.04 *M* Tris pH 7.8, 4×10^{-3} *M* $MgCl_2$, and 10^{-3} *M* $MnCl_2$. The C^{14}-products of the tryptic digestion were identified by paper chromatography as described above.

The identity of the C-terminal end group of the synthesized C^{14}-polypeptide was established by a hydrazinolysis procedure.[28] The polypeptide was treated *in vacuo* at 106° for 20 hr. The amino acid hydrazides and free amino acids in aqueous solution at pH 11.0 were applied to a Dowex-1 column (OH^-), and the free C^{14}-lysine then was eluted with 1.0 *M* acetic acid.

Pancreatic DNase was obtained from the Worthington Biochem. Corp.; pancreatic RNase from Sigma Chem. Co., St. Louis, Mo.; chloramphenicol succinate from Parke, Davis and Co., Detroit, Mich.; and actinomycin D from Merck, Sharp and Dohme Co., Rahway, N. J. Puromycin HCl was the generous gift of Dr. F. H. Bergmann. Protein was determined by a micromodification of the method of Lowry *et al.*[29]

Results.—Characteristics of poly A synthesis: The data of Figure 1 show that C^{14}-AMP incorporation was dependent upon the addition of oligo dT_{13-14} to stage I reaction mixtures, and that C^{14}-AMP incorporation was proportional to the amount of oligo dT added within the range of 2.4 or less mμmoles of nucleotide residues in oligo dT_{13-14}. Incorporation stopped after 30 min of incubation.

The average chain length of the C^{14}-product synthesized in the presence of oligo dT_{13-14} was determined as described in the *Materials and Methods* section. After hydrolyzing the C^{14}-products of the reaction in KOH and separating the mononucleotides by paper chromatography, 339, 308, and 22,900 counts per min of

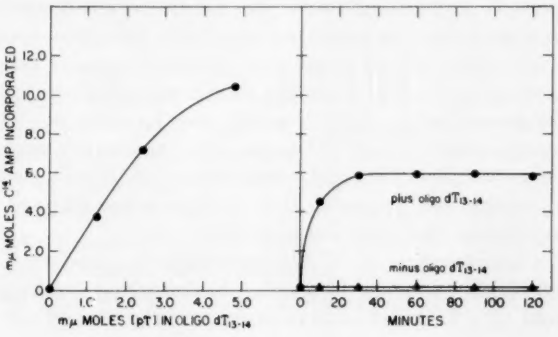

FIG. 1.—Characteristics of C^{14}-poly A synthesis in RNA polymerase (stage I) reaction mixtures. Components of stage I reaction mixtures are described under *Materials and Methods*. In the figure on the left, reaction mixtures were incubated at 37° for 15 min. In the figure on the right, the symbols represent the following: ▲, minus polymer; ●, plus 1.2 mμmoles of (pdT) in oligo dT_{13-14}.

adenosine, adenosine-3′(2′)-5′ diphosphate, and adenosine-3′(2′)-monophosphate, respectively, were found. Thus, oligo dT of chain length 13–14 base residues stimulated the synthesis of poly A of average chain length 60–70 (pA) residues. These results confirm similar observations made by Furth, Hurwitz, and Goldmann[13] and also by Falaschi, Adler, and Khorana.[18]

Characteristics of polylysine synthesis: After incubating stage I reaction mixtures at 37° for 15 min, stage II components were added, and the effect of the newly synthesized poly A upon C^{14}-lysine incorporation into protein was observed. As shown in Figure 2, no increase in C^{14}-lysine incorporation was found in the absence

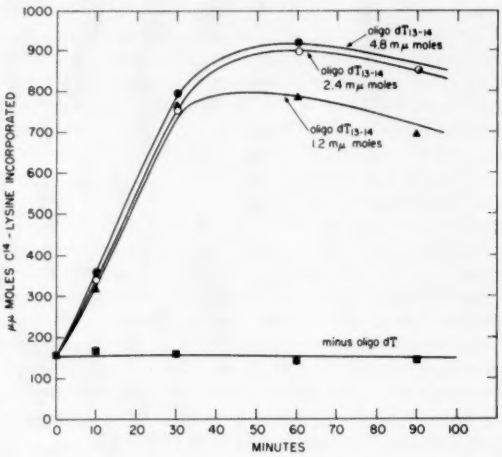

FIG. 2.—Characteristics of oligo dT_{13-14} directed synthesis of C^{14}-polylysine. ■, minus oligo dT; ▲, plus 1.2 mμmoles (pdT) residues in oligo dT_{13-14}; ○, plus 2.4 mμ moles (pdT) residues in oligo dT_{13-14}; ●, plus 4.8 mμmoles (pdT) residues in oligo dT_{13-14}. Components of stage I and stage II reaction mixtures are described under *Materials and Methods*. Stage I reaction mixtures were incubated at 37° for 15 min before addition of stage II components. The length of stage II incubation, in minutes, is shown on the abscissa.

of oligo dT, whereas the addition of 1.2 mμmoles of (pdT) residues in oligo dT_{13-14} stimulated C^{14}-lysine incorporation at a linear and almost optimal rate for 30 min. In separate reaction mixtures, C^{14}-AMP incorporation into poly A was determined at the end of the stage I incubation. No incorporation was observed in the absence of oligo dT, whereas the addition of 1.2 mμmoles of base in oligo dT_{13-14} stimulated the incorporation of 3.8 mμmoles of AMP into poly A, which in turn directed the incorporation of 0.64 mμmoles of C^{14}-lysine into protein. Additional experiments demonstrated that oligo dT directed little or no C^{14}-AMP incorporation into poly A in stage II reaction mixtures. No C^{14}-lysine incorporation into protein was found in stage I reaction mixtures; thus, both incubations were required.

The specificity of amino acid incorporation also was investigated. Poly A was synthesized in stage I reaction mixtures containing oligo dT_{13-14}, and its effect upon the incorporation of 19 C^{14}-amino acids (cysteine not tested) was determined. The incorporation of each C^{14}-amino acid was assayed separately, and all reaction mixtures contained the regular complement of 19 unlabeled amino acids minus the one C^{14}-amino acid present. Only C^{14}-lysine was directed into protein; thus, marked amino acid specificity was observed.

Characteristics of C^{14}-lysine-containing products: Both the C^{14}-products synthesized in stage II reaction mixtures and synthetic polylysine were soluble in cold 10% TCA and could be precipitated by the addition of a sodium tungstate-TCA

solution.[19] Paper chromatography of polylysine as described in the *Materials and Methods* section permits separation of lysine peptides of different chain lengths. Peptides containing approximately 11 or more lysine residues remain at the origin, whereas the mobilities of smaller peptides are as follows: lysine > di > tri > tetra > penta > hexa > hepta > octa > nona > deca-lysine. The scales at the top and bottom of Figure 3 show the chromatographic mobilities of lysine peptides in this system. As seen in Figure 3*A*, when stage I and II reaction mixtures were incubated without oligo dT, little C^{14}-lysine or C^{14}-product remained at the origin after chromatography; however, when incubations were performed in the presence of oligo dT_{13-14}, most of the C^{14}-product synthesized remained at the origin.

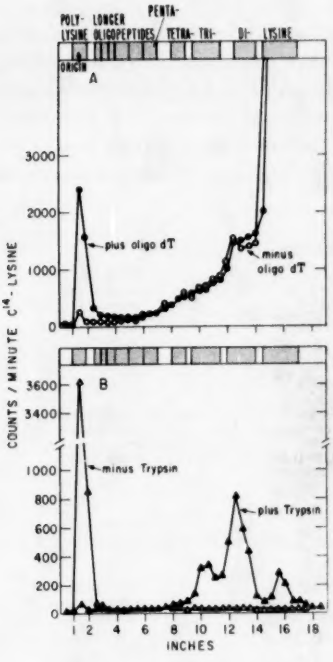

FIG. 3.—Chromatographic analysis of the C^{14}-polylysine in stage I plus stage II reaction mixtures under the direction of oligo dT_{13-14}. Fig. 3*A* depicts the chromatographic analysis of reaction mixtures plus and minus oligo dT_{13-14}. The symbols represent the following: ○, minus oligo dT_{13-14}; ●, plus oligo dT_{13-14}. Reactions were performed as described in the *Materials and Methods* section. After incubation of stage II reaction mixtures at 37° for 30 min, the reactions were terminated by the addition of 2 ml of cold 10% TCA. The supernatant solution was extracted three times with equal volumes of ether, concentrated *in vacuo*, and the residue was compared chromatographically with a partial tryptic digest of chemically synthesized C^{13}-polylysine as described in the *Materials and Methods* section.

In Fig. 3*B*, the C^{14}-products formed in stage I and stage II reaction mixtures were chromatographed as described in the previous paragraph. The C^{14}-product remaining at the origin was eluted and one aliquot was digested with trypsin. The symbols represent the following: △, rechromatography of the isolated product; ▲, tryptic digest of the isolated C^{14}-product. Methods of chromatographic analysis, C^{14}-product isolation, and tryptic digestion are described in the *Materials and Methods* section.

Incubation with trypsin converts polylysine to free, di-, and tri-lysine. The data of Figure 3*B* show that digestion with trypsin converted the isolated C^{14}-product almost quantitatively to peptides which migrated with free, di-, tri-, and tetra-lysine. Tetra-lysine would be expected if digestion were not complete.

In separate experiments, an aliquot of the C^{14}-product, which had been eluted from the origin after paper chromatography, was hydrolyzed completely in HCl, and another aliquot was used to characterize carboxyl-terminal residues. The hydrolysate obtained with HCl was subjected to paper chromatography, and only one C^{14}-spot, having the characteristic mobility of free lysine, was found. In addition, the C^{14}-product was shown to contain carboxyl-terminal radioactivity by a hydrazinolysis method.[28] These experiments strongly suggest that the C^{14}-product is C^{14}-polylysine.

Effect of molecular weight upon the activity of oligo dT: Falaschi, Adler, and Khorana demonstrated[18] that oligo dT chains containing less than 4 (pdT) residues did not stimulate poly A synthesis but that longer chains were stimulatory. The activity was proportional to chain length until maximum stimulation was reached with oligo dT fractions containing 14 or more (pdT) residues per chain. The data of Table 1 are in accord with their findings. Oligo dT fractions containing as few as 6–7 base residues per chain were of sufficient length to stimulate the synthesis of poly A with messenger activity for C^{14}-lysine incorporation. Almost optimal stimulation was obtained with fractions containing 7–8 residues per chain.

Effect of inhibitors upon oligo dT and calf thymus DNA-dependent poly A and polylysine synthesis: Chamberlain and Berg[16] have reported DNA-dependent synthesis of poly A in the presence of only ATP. As shown in Table 2, experiment 2, the poly A product of this reaction stimulates the formation of polylysine. Experiment 1 shows that oligo dT did not stimulate poly A or polylysine synthesis in the absence of RNA polymerase. The data of Table 2 also show

TABLE 1

RELATION BETWEEN OLIGO dT CHAIN LENGTH AND ACTIVITY

Addition	C^{14}-AMP incorporation (mμmoles)	C^{14}-lysine incorporation (mμmoles)
None	0.1	0.075
17 mμmoles oligo dT_{6-7}	1.5	0.237
17 mμmoles oligo dT_{7-8}	6.8	0.387
17 mμmoles oligo dT_{9-11}	13.0	0.406

Components of the reaction mixtures and conditions are described under *Materials and Methods*. C^{14}-AMP incorporation was determined after incubating RNA polymerase (stage I) reaction mixtures containing C^{14}-ATP at 37° for 15 min. C^{14}-lysine incorporation was determined in companion reaction mixtures containing C^{12}- instead of C^{14}-ATP. Stage I and II mixtures were incubated at 37° for 15 and 30 min, respectively.

TABLE 2

EFFECT OF VARIOUS INHIBITORS UPON OLIGO dT AND CALF THYMUS DNA-DEPENDENT POLY A AND POLYLYSINE SYNTHESIS

Expt. no.	Additions	C^{14}-AMP incorporation (mμmoles)	C^{14}-lysine incorporation (mμmoles)
1.	None	0.18	0.045
	+ Oligo dT	4.4	0.232
	" " ", minus RNA polymerase	0.10	0.045
	" " ", + 15 μg DNase	2.0	0.171
	" " ", + 5 μg RNase	4.2	0.059
	" " ", + 20 mμmoles puromycin	2.9	0.079
	" " ", + 50 mμmoles puromycin	2.9	0.062
	" " ", + 90 mμmoles chloramphenicol	4.1	0.161
	" " ", + 180 mμmoles chloramphenicol	4.0	0.150
	" " ", 25 mμmoles actinomycin D	3.3	0.241
2.	None	0.16	0.044
	+ Calf thymus DNA	8.8	0.352
	" " " ", 15 μg DNase	0.34	0.038
	" " " ", 5 μg RNase	8.6	0.028
	" " " ", 60 mμmoles chloramphenicol	8.0	0.352
	" " " ", 20 mμmoles puromycin	8.5	0.063
	" " " ", 25 mμmoles actinomycin D	4.1	0.267

The components of the reaction mixtures and conditions are described in the *Materials and Methods* section. All inhibitors were added to stage I reaction mixtures. RNase and DNase were preincubated at 37° for 20 min with stage I components before the reactions were initiated by the addition of RNA polymerase. Stage I and II mixtures were incubated at 37° for 15 and 30 min, respectively. C^{14}-AMP incorporation was determined after incubating separate RNA polymerase (stage I) reaction mixtures containing C^{14}-ATP. 1.2 mμmoles of (pdT) in oligo dT_{12-16}, or 20 mμmoles of base residues in DNA were added where specified.

the effects of inhibitors upon both incorporations. Paper chromatography did not reveal free (pdT) residues after incubation of oligo dT_{13-14} with pancreatic DNase in stage I reaction mixtures, and preincubation of oligo dT_{13-14} and calf thymus DNA with this enzyme inhibited only moderately the synthesis of C^{14}-poly A and C^{14}-polylysine. C^{14}-Polylysine synthesis was inhibited, in order of decreasing effectiveness, by RNase, puromycin, chloramphenicol, and DNase.

Poly U-directed poly A synthesis: Weiss and his co-workers,[30, 31] and also Krakow and Ochoa[32] have shown that RNA polymerase can catalyze the synthesis of complementary RNA in the presence of either RNA or DNA. Therefore, it seemed feasible to direct poly A synthesis in modified stage I reaction mixtures with RNA (poly U) in place of DNA (oligo dT), and then to determine the activity of the poly A in directing C^{14}-lysine incorporation into protein. As seen in Figure 4,

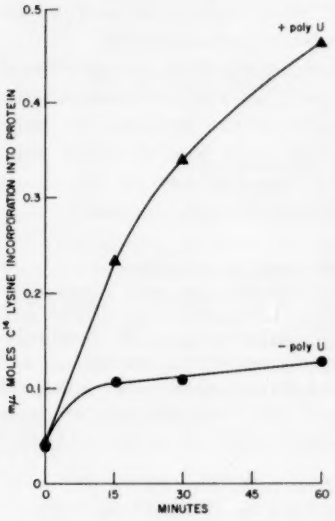

FIG. 4.—Poly U-directed poly A and polylysine synthesis. The components of the stage I reaction mixtures were as follows: 0.04 *M* Tris, pH 7.8; 4×10^{-3} *M* $MgCl_2$; 10^{-3} *M* $MnCl_2$; 2.24×10^{-3} *M* ATP; 6×10^{-3} *M* mercaptoethanol; 20 μg RNA polymerase protein; and 10.2 mμmoles (pU) in poly U where indicated in a total volume of 0.125 ml. After incubation at 37° for 20 min, 0.25 ml of the second stage (protein synthesis) reaction mixture were added to each 0.125 ml stage I reaction mixture. Each 0.25 ml addition contained the following: 0.01 *M* Tris, pH 7.8; 0.014 *M* magnesium acetate; 0.05 *M* KCl; 6×10^{-3} *M* mercaptoethanol; 10^{-3} *M* ATP; 2×10^{-4} *M* each of 19 L-amino acids minus lysine; 2×10^{-5} *M* C^{14}-lysine, and 1.0 mg, preincubated, DNase-treated *E. coli* S-30 protein.[1] Combined 0.375 ml reaction mixtures were incubated at 37° for the stated times.

poly A synthesized from poly U templates stimulated C^{14}-lysine incorporation into protein. In other experiments, no stimulation of C^{14}-lysine incorporation was observed when poly C, rather than poly U, was added to stage I reaction mixtures.

Discussion.—The experiments reported herein have shown that oligo dT_{13-14} directs the synthesis of poly A of average chain-length 60–70 (pA) residues, and the poly A in turn directs the synthesis of polylysine containing 11 or more residues per chain. RNA polymerase is known to catalyze the synthesis of poly A of similar chain length when DNA (preferably denatured) is present and ATP is the only substrate.[16] Our results show that this product also has messenger activity for polylysine synthesis. To account for the chain length of the poly A synthesized in the presence of DNA, Chamberlain and Berg have suggested a "slippage" of poly A along shorter sequences of (pdT) residues in DNA.[16]

Falaschi, Adler, and Khorana[18] have found that oligo dT does not serve as a primer in this system, for (pA) residues were not added to free 3′-hydroxyl ends of

oligo dT chains. Oligo dC was found to stimulate only the incorporation of C^{14}-GMP, whereas oligo dT stimulated C^{14}-AMP incorporation. These results strongly suggest that oligodeoxynucleotides serve as templates rather than as primers.[18] However, exceptions to Watson-Crick base-pairing, such as small stimulations of C^{14}-AMP incorporations by oligo dA, also were reported. Although nucleotide incorporations were almost completely dependent upon the addition of oligo dT under the conditions used in this study, we have detected under other conditions some nucleoside monophosphate incorporations in the absence of oligo dT. The RNA polymerase preparations used in this study were purified 100- to 150-fold, but further purification will be required to determine whether one or more enzymes catalyze the reaction.

Almost optimal stimulation of polylysine synthesis was obtained with oligo dT fractions containing 7–8 residues per chain. Thus, it seems possible that chemically synthesized oligodeoxynucleotides of known sequence may be useful in the determination of nucleotide sequence and polarity of RNA code-words and in the study of control mechanisms related to DNA-directed protein synthesis.

Summary.—In the presence of chemically synthesized oligodeoxythymidylate of chain length 6–14 and ATP, RNA polymerase catalyzes the synthesis of polyadenylate which, in cell-free *E. coli* extracts, directs the synthesis of polylysine. Thymus DNA and polyuridylate also have been used to direct polyadenylate, and then polylysine synthesis. The possible use of chemically synthesized DNA in studies pertaining to the genetic code is briefly discussed.

* The following abbreviations are used: poly d(AT), poly (deoxyadenylate-deoxythymidylate) copolymer; poly dG, polydeoxyguanylate; poly dC, polydeoxycytidylate; poly A, polyadenylate; poly U, polyuridylate; poly C, polycytidylate; oligo dT, oligodeoxythymidylate; pdT, deoxythymidine-5′-phosphate residues in oligo dT; pA, adenosine-5′-phosphate residues in poly A; AMP, adenosine-5′-phosphate; ATP, adenosine-5′-triphosphate; PP, pyrophosphate; subscript following oligonucleotides (example: oligo dT_{13-14}) refers to the number of nucleotide residues per molecule; tris, tris(hydroxymethyl) aminomethane; PEP, phosphoenol pyruvate, potassium salt; TCA, trichloroacetic acid; PPO, 2,5-diphenyloxazole; POPOP, 1,4-bis-2′-(5′-phenyloxazolyl) benzene.

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THE INDEPENDENCE OF THE CONTINUUM HYPOTHESIS

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This is the first of two notes in which we outline a proof of the fact that the Continuum Hypothesis cannot be derived from the other axioms of set theory, including the Axiom of Choice. Since Gödel[3] has shown that the Continuum Hypothesis is consistent with these axioms, the independence of the hypothesis is thus established. We shall work with the usual axioms for Zermelo-Fraenkel set theory,[2] and by Z-F we shall denote these axioms without the Axiom of Choice, (but with the Axiom of Regularity). By a model for Z-F we shall always mean a collection of actual sets with the usual ϵ-relation satisfying Z-F. We use the standard definitions[3] for the set of integers ω, ordinal, and cardinal numbers.

THEOREM 1. *There are models for Z-F in which the following occur:*

(1) *There is a set a, $a \subseteq \omega$ such that a is not constructible in the sense of reference 3, yet the Axiom of Choice and the Generalized Continuum Hypothesis both hold.*

(2) *The continuum (i.e., $\mathcal{P}(\omega)$ where $\mathcal{P}$ means power set) has no well-ordering.*

(3) *The Axiom of Choice holds, but $\aleph_1 \neq 2^{\aleph_0}$.*

(4) *The Axiom of Choice for countable pairs of elements in $\mathcal{P}(\mathcal{P}(\omega))$ fails.*

Only part 3 will be discussed in this paper. In parts 1 and 3 the universe is well-ordered by a single definable relation. Note that 4 implies that there is no simple ordering of $\mathcal{P}(\mathcal{P}(\omega))$. Since the Axiom of Constructibility implies the Generalized Continuum Hypothesis,[3] and the latter implies the Axiom of Choice,[5] Theorem 1 completely settles the question of the relative strength of these axioms.

Before giving details, we sketch the intuitive ideas involved. The starting point is the realization[1, 4] that no formula $\mathfrak{a}(x)$ can be shown from the axioms of Z-F to have the property that the collection of all x satisfying it form a model for Z-F in which the Axiom of Constructibility ($V = L$,[3]) fails. Thus, to find such models, it seems natural to strengthen Z-F by postulating the existence of a set which is a

model for Z-F, thus giving us greater flexibility in constructing new models. (In the next paper we discuss how the question of independence, as distinct from that of models, can be handled entirely within Z-F.) The Löwenheim-Skolem theorem yields the existence of a countable model $\mathfrak{M}$. Let $\aleph_1, \aleph_2$, etc., denote the corresponding cardinals in $\mathfrak{M}$. Since $\mathfrak{M}$ is countable, there exist distinct sets $a_\delta \subseteq \omega$, $0 \leq \delta \leq \aleph_2$. Put $V = \{\langle a_\delta, a_{\delta'}\rangle | \delta < \delta'\}$. We form the model $\mathfrak{N}$ "generated" from $\mathfrak{M}$, a_δ, and V and hope to prove that in $\mathfrak{N}$ the continuum has cardinality at least $\aleph_2$. Of course, $\mathfrak{N}$ will contain many new sets and, if the a_δ are chosen indiscriminately, the set $\aleph_2$ (in $\mathfrak{M}$) may become countable in $\mathfrak{N}$. Rather than determine the a_δ directly, we first list all the countably many possible propositions concerning them and decide in advance which are to be true. Only those properties which are true in a "uniform" manner for "generic" subsets of ω in $\mathfrak{M}$ shall be true for the a_δ in $\mathfrak{N}$. For example, each a_δ contains infinitely many primes, has no asymptotic density, etc. If the a_δ are chosen in such a manner, no new information will be extracted from them in $\mathfrak{N}$ which was not already contained in $\mathfrak{M}$, so that, e.g., $\aleph_2$ will remain the second uncountable cardinal. The primitive conditions $n \in a_\delta$ are neither generically true nor false, and hence must be treated separately. Only when given a finite set of such conditions will we be able to speak of properties possibly being forced to hold for "generic" sets. The precise definition of forcing will be given in Definition 6.

From now on, let $\mathfrak{M}$ be a fixed countable model for Z-F, satisfying $V = L$, such that $x \in \mathfrak{M}$ implies $x \subset \mathfrak{M}$. If $\mathfrak{M}'$ is a countable model without this property, define Ψ by transfinite induction on the rank of x, so that $\Psi(x) = \{y | \exists z \in \mathfrak{M}', z \in x, \Psi(z) = y\}$; the image $\mathfrak{M}$ of $\mathfrak{M}'$ under Ψ is isomorphic to $\mathfrak{M}'$ with respect to $\in$ and satisfies our requirement. Thus, the ordinals in $\mathfrak{M}$ are truly ordinals. Let $\tau > 1$ be a fixed ordinal in $\mathfrak{M}$, $\aleph_\tau$ the corresponding cardinal in $\mathfrak{M}$, and let a_δ, $0 \leq \delta < \aleph_\tau$ be subsets of ω, not necessarily in $\mathfrak{M}$, $V = \{\langle a_\delta, a_{\delta'}\rangle | \delta < \delta'\}$.

LEMMA 1. *There exist unique functions j, K_1, K_2, N, from ordinals to ordinals definable in $\mathfrak{M}$ such that*

(1) *$j(\alpha + 1) > j(\alpha)$ and for all β such that $j(\alpha) + 1 < \beta < j(\alpha + 1)$ the map $\beta \to (N(\beta), K_1(\beta), K_2(\beta))$ is a 1-1 correspondence between all such β, and the set of all triples (i, γ, δ), $1 \leq i \leq 8$, $\gamma < j(\alpha)$, $\delta < j(\alpha)$. Furthermore, this map is order-preserving if the triples are given the natural ordering S (Ref. 3, p. 36).*

(2) *$j(0) = 3\aleph_\tau + 1$, $j(\alpha) = sup\{j(\beta) | \beta < \alpha\}$ if α is a limit ordinal.*

(3) *$N(j(\alpha)) = 0$, $N(j(\alpha) + 1) = 9$, $K_i = 0$ for these values.*

(4) *$N(\alpha)$, $K_i(\alpha)$ are zero if $\alpha \leq 3\aleph_\tau$.*

(5) *If β is as above, and $N(\beta) = i$, put $J(i, K_1(\beta), K_2(\beta), j(\alpha)) = \beta$. Also put $I(\beta) = j(\alpha)$.*

Definition 1: For α an ordinal in $\mathfrak{M}$, define F_α by means of induction as follows:

(1) $F_\alpha = \alpha$ if $\alpha \leq \omega$.

(2) For $\omega < \alpha < 3\aleph_\tau$, let F_α successively enumerate a_δ, the unordered pairs $(a_\delta, a_{\delta'})$ and the ordered pairs $\langle a_\delta, a_{\delta'}\rangle$ in any standard manner (e.g., the ordering R on pairs of ordinals def. 7.81[3]).

(3) For $\alpha = 3\aleph_\tau$, $F_\alpha = V$.

(4) For $\alpha > 3\aleph_\tau$, if $K_1(\alpha) = \beta$, $K_2(\alpha) = \gamma$
if $N(\alpha) = 0$, $F_\alpha = \{F_{\alpha'} | \alpha' < \alpha\}$
if $1 \leq i = N(\alpha) \leq 8$, $F_\alpha = \mathfrak{F}_i(F_\beta, F_\gamma)$

where $\mathfrak{F}_i$ are defined as follows (Def. 9.1[3]):

$$\begin{aligned}
\mathfrak{F}_1(x, y) &= \{x, y\} \\
\mathfrak{F}_2(x, y) &= \{\langle s, t\rangle \mid s \in t, \langle s, t\rangle \in x\} \\
\mathfrak{F}_3(x, y) &= x - y \\
\mathfrak{F}_4(x, y) &= \{\langle s, t\rangle \mid \langle s, t\rangle \in x, t \in y\} \\
\mathfrak{F}_5(x, y) &= \{s \mid s \in x, \exists t\, \langle t, s\rangle \in y\} \\
\mathfrak{F}_6(x, y) &= \{\langle s, t\rangle \mid \langle s, t\rangle \in x, \langle t, s\rangle \in y\} \\
\mathfrak{F}_7(x, y) &= \{\langle r, s, t\rangle \mid \langle r, s, t\rangle \in x, \langle t, r, s\rangle \in y\} \\
\mathfrak{F}_8(x, y) &= \{\langle r, s, t\rangle \mid \langle r, s, t\rangle \in x, \langle r, t, s\rangle \in y\}
\end{aligned}$$

if $N(\alpha) = 9$, $F_\alpha = \{F_{\alpha'} \mid \alpha' < \alpha, N(\alpha') = 9\}$. We also define $T_\alpha = \{F_\beta \mid \beta < \alpha\}$.

We have introduced the case $N = 9$ for the convenience of later arguments. It ensures that the ordinals in $\mathfrak{M}$ are listed among the F_α in a canonical manner. Observe that since $V = L$ holds in $\mathfrak{M}$, it is not difficult to see that this implies $\mathfrak{M} \subseteq \mathfrak{N}$.

Denote by $\mathfrak{N}$, the set of all F_α for $\alpha \in \mathfrak{M}$. Note that in $\mathfrak{N}$ each F_α is a collection of preceding F_β. We shall often write α in place of F_α if $\alpha \leq \omega$, and a_δ in place of $F_{\delta+\omega+1}$, etc., if there is no danger of confusion. If $N(\alpha) = 9$, then the set F_α is defined independently of a_δ. We shall now examine statements concerning F_α before the a_δ are actually determined, and thus the F_α for a while shall be considered as merely formal symbols.

Definition 2: (1) $x \in y$, $x \in F_\alpha$, $F_\alpha \in x$, $F_\alpha \in F_\beta$ are formulas; (2) if φ and ψ are formulas, so are $\neg\varphi$ and $\varphi \,\&\, \psi$; and (3) only (1) and (2) define formulas.

Definition 3: A *Limited Statement* is a formula $\mathfrak{a}(x_1, \ldots, x_n)$ in which all variables are bound by a universal quantifier $(x_i)_\alpha$ or an existential quantifier $\exists_\alpha x_i$ placed in front of it, where α is an ordinal in $\mathfrak{M}$. An *Unlimited Statement* is the same except that no ordinals are attached to the quantifiers.

Our intention is that the variable x in $(x)_\alpha$ or $\exists_\alpha x$ is restricted to range over all F_β with $\beta < \alpha$. The symbol $=$ is not used since by means of the Axiom of Extensionality it can be avoided. We only consider statements in prenex form. Since it is clear how to reduce negations, conjunctions, etc., of such statements to prenex form, we shall not do so if there is no risk of confusion.

Definition 4: The rank of a limited statement $\mathfrak{a}$ is (α, r) if r is the number of quantifiers and α is the least ordinal such that for all β, $\beta < \alpha$ if F_β occurs in $\mathfrak{a}$, and $\beta \leq \alpha$ if $(x)_\beta$ or $\exists_\beta x$ occurs in $\mathfrak{a}$. We write $(\alpha, r) < (\beta, s)$ if $\alpha < \beta$ or $\alpha = \beta$ and $r < s$.

Thus, if rank $\mathfrak{a} = (\alpha, r)$, $\mathfrak{a}$ can be formulated in $\{F_\beta \mid \beta < \alpha\}$.

Definition 5: Let P denote a *finite* set of conditions of the form $n \in a_\delta$ or $\neg\, n \in a_\delta$ such that no condition and its negation are both included.

In the following definition, which is the key point of the paper, we shall define a certain concept for all limited statements by means of transfinite induction. The well-ordering we use is not, however, precisely the corresponding ordering of the ranks, but requires a slight modification. We say $\mathfrak{a}$ is of type $\mathfrak{R}$, if rank $\mathfrak{a} = (\alpha + 1, r)$, $(x)_{\alpha+1}$ and $\exists_{\alpha+1} x$ do not occur in $\mathfrak{a}$, and no expression of the form $F_\alpha \in (\cdot)$ occurs in $\mathfrak{a}$. We order the limited statements by saying, if rank $\mathfrak{a} = (\alpha, r)$ and rank $\mathfrak{b} = (\beta, s)$, $\mathfrak{a}$ precedes $\mathfrak{b}$ if and only if rank $\mathfrak{a}$ < rank $\mathfrak{b}$, unless $\alpha = \beta$ and one of

the two statements $\mathfrak{a}$, $\mathfrak{b}$ is of type $\mathfrak{R}$ and the other is not of type $\mathfrak{R}$, in which case the former precedes the latter.

Definition 6: By induction, we define the concept of "P forces $\mathfrak{a}$" as follows:

I. If $r > 0$, P forces $\mathfrak{a} = (x)_\alpha \mathfrak{b}(x)$ if for all $P' \supset P$, P' does not force $\neg\mathfrak{b}(F_\beta)$ for $\beta < \alpha$. P forces $\exists_\alpha x \mathfrak{b}(x)$ if for some $\beta < \alpha$, P forces $\mathfrak{b}(F_\beta)$.

II. If $r = 0$, and $\mathfrak{a}$ has propositional connectives, P forces $\mathfrak{a}$ if for each component $F_\alpha \in F_\beta$ or $\neg F_\alpha \in F_\beta$ appearing in $\mathfrak{a}$, these, by case III of this definition, are forced to be true or their negations are forced to be true so that in the usual sense of the propositional calculus $\mathfrak{a}$ is true.

III. If $\mathfrak{a}$ is of the form $F_\alpha \in F_\beta$ or $\neg F_\alpha \in F_\beta$, we define P forces $\mathfrak{a}$ as follows:

(*i*) If $\alpha, \beta \leq 3\aleph_\tau$, then $\mathfrak{a}$ must hold as a formal consequence of P, i.e., P forces $\mathfrak{a}$, if $\mathfrak{a}$ is true whenever a_δ are distinct subsets of ω, satisfying P, different from any integer and ω.

(*ii*) $\neg F_\alpha \in F_\alpha$ is always forced.

(*iii*) If $\alpha < \beta$, $N(\beta) = i < 9$, $\beta > 3\aleph_\tau$, P forces $\mathfrak{a}$, where $\mathfrak{a} \equiv F_\alpha \in F_\beta$ or $\neg F_\alpha \in F_\beta$, if P forces ψ_i or $\neg \psi_i$, respectively, where ψ_i is the limited statement expressing the definition of F_β. That is, if $K_1(\beta) = \gamma$, $K_2(\beta) = \delta$:

(0) ψ_0 is vacuous and always forced.

(1) $\psi_1 \equiv F_\alpha = F_\gamma \vee F_\alpha = F_\delta$.

(2) $\psi_2 \equiv \exists_\beta x \exists_\beta y\ (F_\alpha = \langle x, y\rangle \,\&\, x \in y \,\&\, F_\alpha \in F_\gamma)$.

(3) $\psi_3 \equiv F_\alpha \in F_\gamma \,\&\, \neg F_\alpha \in F_\delta$.

(4) $\psi_4 \equiv \exists_\beta x \exists_\beta y\ (F_\alpha = \langle x, y\rangle \,\&\, F_\alpha \in F_\gamma \,\&\, y \in F_\delta)$.

(5) $\psi_5 \equiv \exists_\beta x\ (F_\alpha \in F_\gamma \,\&\, \langle x, F_\alpha\rangle \in F_\delta)$.

(6), (7), (8), similarly.

Here the use of ordered pairs must eventually be replaced by their definition, and the use of equality in $x = y$ is replaced by $(z)_\beta(z \in x \leftrightarrows z \in y)$.

(*iv*) If $\alpha < \beta$, $N(\beta) = 9$, $\beta > 3\aleph_\tau$, P forces $\mathfrak{a} \equiv F_\alpha \in F_\beta$ if for some $\beta' < \beta$, $N(\beta') = 9$, P forces $F_\alpha = F_{\beta'}$. P forces $\neg F_\alpha \in F_\beta$, if for all $\beta' < \beta$, $N(\beta') = 9$ and all $P' \supset P$, P' does not force $F_\alpha = F_{\beta'}$. Again the symbol $=$ is treated as before.

(*v*) If $\alpha > \beta$, we reduce the case $F_\alpha \in F_\beta$ to cases (*iii*) and (*iv*) treated above. We say P forces $F_\alpha \in F_\beta$ if for some $\beta' < \beta$, P forces $F_{\beta'} \in F_\beta$ and P forces $F_\alpha = F_{\beta'}$ (i.e., $(x)_\alpha(x \in F_\alpha \leftrightarrows x \in F_{\beta'})$ which is a statement of type $\mathfrak{R}$ and hence precedes $F_\alpha \in F_\beta$). We say P forces $\neg F_\alpha \in F_\beta$ if for all $\beta' < \beta$ and $P' \supset P$, P' does not force both $F_{\beta'} \in F_\beta$ and $F_{\beta'} = F_\alpha$.

The most important part of Definition 6 is I, the other parts are merely obvious derivatives of it.

Definition 7: If $\mathfrak{a}$ is an unlimited statement with r quantifiers, we define "P forces $\mathfrak{a}$" by induction on r. If $r = 0$, then $\mathfrak{a}$ is a limited statement. If $\mathfrak{a} \equiv (x)\ \mathfrak{b}(x)$, P forces $\mathfrak{a}$, if for all $P' \supset P$, and α, P' does not force $\neg\ \mathfrak{b}(F_\alpha)$. If $\mathfrak{a} \equiv \exists x\ \mathfrak{b}(x)$, P forces $\mathfrak{a}$ if for some α, P forces $\mathfrak{b}(F_\alpha)$.

In the proofs of Lemmas 2, 3, 4, and 5, we keep the same well-ordering on limited statements as in Definition 6, and proceed by induction.

LEMMA 2. *P does not force $\mathfrak{a}$ and $\neg\,\mathfrak{a}$, for any $\mathfrak{a}$ and P.*

Proof: Let $\mathfrak{a}$ be a limited statement with r quantifiers. If $r > 0$, and P forces both $\exists_\alpha x\ \mathfrak{b}(x)$ and $(x)_\alpha \neg\ \mathfrak{b}(x)$, then P must force $\mathfrak{b}(F_\beta)$ for $\beta < \alpha$ which means P cannot force $(x)_\alpha \neg\ \mathfrak{b}(x)$. Case II of Definition 6 will clearly follow from case III. Parts (*i*) and (*ii*) are trivial. If $\mathfrak{a}$ is in part (*iii*), then P forces $\mathfrak{a}$ if and only if P

forces a statement of lower rank and in this case the lemma follows by induction. In part (*iv*), if P forces $F_\alpha \in F_\beta$, then for some $\beta' < \beta$, $N(\beta') = 9$, and P forces $F_\alpha = F_{\beta'}$ which means P can not force $\neg F_\alpha \in F_\beta$. In part (*v*) if P forces $F_\alpha \in F_\beta$, for some $\beta' < \beta$, P forces $F_{\beta'} \in F_\beta$ and $F_\alpha = F_{\beta'}$ which again violates P forcing $\neg F_\alpha \in F_\beta$. If $\mathfrak{a}$ is an unlimited statement, the lemma follows in the same manner by induction on the number of quantifiers.

LEMMA 3. *If P forces $\mathfrak{a}$ and $P' \supset P$, then P' forces $\mathfrak{a}$.*

Proof by induction as in Lemma 2.

LEMMA 4. *For any statement $\mathfrak{a}$ and condition P, there is $P' \supset P$ such that either P' forces $\mathfrak{a}$ or P' forces $\neg \mathfrak{a}$.*

Proof: Let $\mathfrak{a}$ be a limited statement with r quantifiers. If $r > 0$ and P does not force $\mathfrak{a} \equiv (x)_\alpha \mathfrak{b}(x)$, then for some $P' \supset P$, P' forces $\neg \mathfrak{b}(F_\beta)$, $\beta < \alpha$, which means P' forces $\neg \mathfrak{a}$. If $r = 0$, we may restrict ourselves to III, for if we enumerate the components of $\mathfrak{a}$, by defining a finite sequence P_n, $P_0 = P$ and $P_{n+1} \supset P_n$ we may successively force each component or its negation so that finally either $\mathfrak{a}$ or $\neg \mathfrak{a}$ is forced. Again, cases (*i*) and (*ii*) are trivially disposed of. Case (*iii*) is handled by induction as before. If $\mathfrak{a} \equiv F_\alpha \in F_\beta$ is in case (*iv*) then if P does not force $\neg \mathfrak{a}$, for some $P' \supset P$ and $\beta' < \beta$, $N(\beta') = 9$, P' forces $F_\alpha = F_{\beta'}$ so P' forces $\mathfrak{a}$. If $\mathfrak{a} \equiv F_\alpha \in F_\beta$ is in case (*v*) if P does not force $\neg \mathfrak{a}$, then for some $P' \supset P$, $\beta' < \beta$, P' forces $F_{\beta'} \in F_\beta$ and $F_{\beta'} = F_{\alpha'}$ hence P' forces $\mathfrak{a}$. Unlimited statements are handled as before.

Definition 8: Enumerate all statements $\mathfrak{a}_n$, both limited and unlimited, and all ordinals α_n in $\mathfrak{M}$. Define P_{2n} as the first extension of P_{2n-1} which forces either $\mathfrak{a}_n$ or $\neg \mathfrak{a}_n$. Define P_{2n+1} as the first extension of P_{2n} which has the property that it forces $F_\beta \in F_{\alpha_n}$ where β is the least possible ordinal for which there exists such an extension of P_{2n}, whereas if no such β exists, put $P_{2n+1} = P_{2n}$.

The sequence P_n is not definable in $\mathfrak{M}$. Since all statements of the form $n \in a_\delta$ are enumerated, P_n clearly approach in an obvious sense, sets a_δ of integers. With this choice of a_δ, let $\mathfrak{N}$ be defined as the set of all F_α defined by Definition 1.

LEMMA 5. *All statements in $\mathfrak{N}$ which are forced by some P_n are true in $\mathfrak{N}$ and conversely.*

Proof: Let $\mathfrak{a}$ be a limited statement with r quantifiers. If $r > 0$, then if P_n forces $(x)_\alpha \mathfrak{b}(x)$, if $\beta < \alpha$, then some P_m must force $\mathfrak{b}(F_\beta)$ since no P_m can force $\neg \mathfrak{b}(F_\beta)$. By induction we have that $\mathfrak{b}(F_\beta)$ holds, so that $(x)_\alpha \mathfrak{b}(x)$ holds in $\mathfrak{N}$. If P_n forces $\exists_\alpha x\, \mathfrak{b}(x)$, for some $\beta < \alpha$, P_n forces $\mathfrak{b}(F_\beta)$ so by induction $\mathfrak{b}(F_\beta)$ holds and hence $\exists_\alpha x\, \mathfrak{b}(x)$ holds in $\mathfrak{N}$. Case II will clearly follow from case III and (*i*) and (*ii*) are trivial. If $\mathfrak{a}$ is $F_\alpha \in F_\beta$ or $\neg F_\alpha \in F_\beta$ in case (*iii*) then if P_n forces $\mathfrak{a}$, P_n forces precisely the statement which because of the definition of F_β is equivalent to $\mathfrak{a}$. In case (*iv*) if P_n forces $F_\alpha \in F_\beta$, for some $\beta' < \beta$, $N(\beta') = 9$, P_n forces $F_{\beta'} = F_\alpha$, which therefore holds by induction in $\mathfrak{N}$. If P_n forces $\neg F_\alpha \in F_\beta$, then for each $\beta' < \beta$, $N(\beta') = 9$, $F_\alpha = F_{\beta'}$ is not forced by any P_m so some P_m must force $F_\alpha \neq F_{\beta'}$ which proves $\neg F_\alpha \in F_\beta$ holds in $\mathfrak{N}$. Similarly for case (*v*) and for unlimited statements. Since every statement or its negation is forced eventually, the converse is also true.

Lemma 5 is the justification of the definition of forcing since we can now throw back questions about $\mathfrak{N}$ to questions about forcing which can be formulated in $\mathfrak{M}$.

In the next paper, we shall prove that $\mathfrak{N}$ is a model for Z-F in which part 3 of Theorem 1 holds.

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A SPECIFIC COMPLEMENT-FIXING ANTIGEN PRESENT IN SV40 TUMOR AND TRANSFORMED CELLS

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Many experimental tumors, both carcinogen-induced[1] and virus-induced,[2–5] contain new cellular antigens, generally demonstrable by transplant rejection procedures. Huebner *et al.*[6] first demonstrated the presence of new, noninfectious, complement-fixing (CF) antigens clearly under viral genetic control, in adenovirus-induced tumors in hamsters and rats.

A new transplantation antigen(s) has been found in SV40-induced hamster tumors,[7–9] but it is not established whether its synthesis is under viral or host cell genetic control. This paper presents evidence for a new CF antigen in SV40 tumors and transformed tissue culture cells, formed from information contained within the viral genome. Preliminary results of these studies were presented by Huebner *et al.*[6]

Materials and Methods.—The CF procedure was identical with that used by Huebner *et al.*;[6] this is a Bengtson procedure done in microplates using overnight fixation at 4°C, with two exact units of complement. Tumor extracts consisted of 10% suspensions in Eagle's basal medium, clarified by centrifugation at 2500 rpm for 30 min, and stored at −60°C. The extracts were tested for antigens only if the undiluted extract was not anticomplementary (AC). The primary SV40-induced hamster tumors used in these studies are from experiments described in detail elsewhere.[10] Tumor extracts used as standard CF antigens were selected for having high titer reactivity with sera from tumorous hamsters.

Suspensions of both normal and transformed tissue culture cells of various species,[11] as well as tissue culture cells from a variety of hamster tumors, were prepared in the following manner. Cells grown in 32-oz Blake bottles were washed with phosphate-buffered saline (PBS) (pH 7.2), scraped off the glass with a rubber policeman, centrifuged at 150 *g* for 8 min, and resuspended in 9 volumes of PBS. These suspensions were stored at −60°C before use.

"Viral antigen" was prepared by inoculating SV40 strain 776[12] into a continuous tissue culture cell line (strain BSC-1) of African green monkey kidney (AGMK) at a multiplicity of about 10^{-4}, and harvesting the cells and fluid together when cytopathogenicity was maximal. The cell suspension was stored at −20°C, thawed, and used without further processing.[13]

Four types of sera were used as standard reagents; to avoid undue heterogeneity of antibodies, sera of individual animals generally were used: (1) serum from tumorous hamsters, selected for having high CF antibody titer against SV40 tumor extracts, and no reaction with viral antigen; (2) serum from similar hamsters, but having high CF antibody titers for both tumor extracts and

TABLE 1

REPRESENTATIVE PATTERNS OF CF ANTIBODY RESPONSES TO TUMOR AND VIRAL ANTIGEN

	Reciprocals of Antibody Titer Versus Undiluted Antigen and Antigen Titer Versus 1:20 Serum										
	Serum of hamster with primary tumor; #5299		Serum of hamster with transformed cell tumor from clone 3-3*; #5246		Serum of hamster with transformed cell tumor from clone 3-3; #5074		SV40 immune monkey serum #2159		Hamster hyperimmune anti-SV40 serum #6141		
Antigen	Antibody titer	Antigen titer	Antibody titer	Antigen titer	Antibody titer	Antigen titer	Antibody titer	Antigen titer	Antibody titer	Antigen titer	Virus titer ($TCID_{50}$/0.1 ml)
Primary SV-40 hamster tumor #1369	≥80	8	≥80	16	≥80	≥16	40	8	0†	0	$<10^0$
1st passage transplant of primary SV40 hamster tumor #2007	≥80	8	≥80	16	≥80	≥16	20	8	0	0	$<10^0$
Tumor from transformed hamster kidney cell clone 3-3; #1918	≥80	8	≥80	8	≥80	8	(20)‡	1	0	0	$<10^0$
SV40 virus (BSC-1 TC§)	0	0	10	≥ 4	0	0	640	4	20	4	$10^{9.0}$
Control (BSC-1 TC)			0	0			0	0	0	0	
Polyoma-induced hamster sarcoma	0	0	0	0	0	0	0	0	0	0	

* Clone 3-3 = clonal line derived from THK-1 line of SV40 transformed hamster kidney cells.[17]
† 0 = <20 serum antibody titer or <1 antigen titer.
‡ (20) = partial reaction at 1:20.
§ TC = tissue culture.

viral antigen; (3) convalescent serum (#2159) from a monkey inoculated with SV40 virus;[14] and (4) serum of weanling hamsters hyperimmunized with SV40 virus grown in AGMK cultures.

Tests for CF antigens in tumor extracts were done by testing against hamster serum diluted (generally 1:10 or 1:20) to contain 4–16 units of antitumor antibody, and against the monkey immune serum at a dilution of 1:20, which corresponded to 1–2 units of antibody reactive with tumor extracts. The same monkey serum was used at a dilution of 1:80 to test for viral antigen in AGMK harvests; this dilution of the serum contained 8 units of antiviral antibody (*vide infra*). Hamster sera were screened for CF antibody at a dilution of 1:5 or 1:10, using 8 units of tumor antigen or 4 units of viral antigen.

The methods used for virus assay and neutralization tests have been described elsewhere.[10]

Ultracentrifugal fractionation of antigens was done by the following procedure. Frozen material was thawed and reclarified by centrifugation at 2,500 rpm for 15 min. The supernatant fluid was centrifuged at 30,000 rpm (59,310 *g* av) for 2 hr in a Spinco no. 40 rotor at 4°C; the upper and lower halves of the supernatant fluid were saved separately. The pellet was rinsed with Eagle's basal medium, and resuspended to one-tenth volume in PBS.

Results.—Antigen in SV40 hamster tumors: Of 35 extracts of primary SV40-induced sarcomas, 32 (91%) reacted in CF with late sera from hamsters with primary tumors. Three transplanted primary tumors and seven tumors induced by inoculation of transformed hamster kidney or hamster embryo tissue culture cells all reacted with these sera. The majority of primary, transplanted, and transformed cell-induced tumors also reacted with the 1:20 dilution of the SV40 monkey immune serum. The quantity of antigen reacting with the hamster antiserum was not related to size of tumor; primary tumors less than 1 cm in diameter were as often positive, and in as high a titer, as medium-sized and large tumors. Likewise, there was no apparent relationship to presence or titer of extractable infectivity in the tumor.

Table 1 shows representative titration results with the various antisera. The three SV40 tumors showed complete reciprocal cross-reactions, indicating that the same antigen(s) was present in primary, transplanted, and clone-induced tumors. None of these tumors contained demonstrable infectivity. Although the tumor extracts reacted with the monkey immune serum, the antibody titer obtained was far lower than the titer of antiviral antibodies, and there was no reaction between these extracts and hyperimmune hamster SV40 antiserum.

Presence of antigen in transformed cells of various species origin: SV40 infection induces a proliferative response accompanied by alterations in morphology and growth pattern of tissue culture cells derived from a number of mammalian species.[11] The continuous cell lines of transformed cells derived from these cultures of various species origin provided a unique opportunity to approach the question of whether the new antigen detected in hamster tumors was formed in response to information contained in the host or in the viral genome. As seen in Table 2, "transformed" cells of hamster, rabbit, Swiss mouse, C3H mouse, and porcine origin reacted with the SV40 hamster tumor and monkey immune sera in a fashion similar to tumor extracts. The antigen was also present, in low titer, in a suspension of second passage cells from a fetal bovine kidney culture chronically infected with SV40, but showing no evidence of transformation; the supernatant culture fluid at this passage level contained only trace amounts of virus.

A variety of control antigens gave no significant reaction with these sera; it is particularly noteworthy that the positive hamster serum did not react with Forssmann antigen in guinea pig kidney, although the monkey serum gave a slight reaction. Most of the positive and control hamster antigens reacted with a low dilution

TABLE 2

PRESENCE OF "TUMOR" ANTIGEN IN CELLS OF VARIOUS SPECIES INFECTED WITH SV40

Antigen		CF Antigen Titer Versus Indicated Serum				
Species	Material	Primary SV40 hamster tumor serum #5586 1:10 (16 antibody units)	SV40 immune monkey serum #2159 1:20	Primary adeno 12 hamster tumor serum 1:10	Polyoma hamster tumor serum 1:10	SV40 negative African green monkey serum 1:20
Hamster	SV40 primary tumor	8	8	0[a]	0	
	TC[b] cells from SV40 primary tumor	2	0	0	0	0
	SV40 transformed TC cells-THK-1[c] (P-59)[d]	8	≥4	0	0	0
	SV40 transformed TC cells clone 3-3[e] (P-34)	≥8	2	0	0	0
	SV40 transformed TC cells clone 4-1[e] (P-37)	≥8	≥4	0	0	0
	SV40 transformed TC cells clone 4-2[e] (P-36)	≥8	≥4	0	0	0
	SV40 transformed TC cells[f] #3775	≥8	≥4	0	0	0
	Controls[h]	All 0	All 0			
Rabbit	SV 40 "transformed" kidney TC (P-12)	≥8	2	0	0	0
	Normal kidney TC (P-1)	0	0			
Swiss mouse	SV 40 "transformed" kidney TC (P-16)	≥8	≥4	0	0	0
	Normal kidney TC (P-1)	0	0			
C3H mouse	SV40 "transformed" kidney TC (P-4)	≥8	≥4	0	0	0
	Normal kidney TC (P-1)	0	0			
Pig	SV40 "transformed" kidney TC (P-2)	≥8	≥4	0	0	0
	Normal kidney TC (P-2)	0	0			
Cow	SV40 infected kidney TC (P-2)	2	0			
	Normal kidney TC (P-2)	0	0			
	Bovine papilloma transformed cells (P-23)[g]	0	0			
Guinea pig	Normal kidney suspension	0	1			

[a] 0 = no reaction with undiluted antigen.
[b] TC = tissue culture.
[c] THK-1 = continuous cell line of SV40 transformed hamster kidney cells.[16]
[d] P = passage level.
[e] Clonal lines derived from THK-1 line.[17]
[f] Tissue culture line of cells from tumors induced with SV40 transformed hamster embryo cells; these tumors were kindly supplied by Dr. Joseph Melnick.
[g] Continuous cell line of bovine conjunctiva transformed by bovine papilloma virus.[20]
[h] Normal carcass; normal kidney TC; primary polyoma tumor; polyoma primary tumor TC cells (Stanton); polyoma-transformed hamster embryo cells (Dulbecco); adenovirus 12 tumor; adenovirus 12 tumor TC cells P-26 (Schlesinger); adenovirus 18 tumor; Rous tumor.

of rabbit antisheep erythrocyte serum, indicating the presence of the Forssmann antigen in many of these cell lines;[15] however, all antigens of the other species had negative to partial reactions with this serum. The positively reacting antigens did not react with sera of hamsters with other virus-induced tumors or with a monkey serum negative for SV40 antibodies.

Comparison of some properties of tumor and viral antigens: Although the reaction of tumor extracts and transformed cells with the antiviral monkey serum suggested that the tumors contained antigens in common with the viral antigen preparation, the properties of the two types of antigen differed in at least two important respects. Firstly, antigens in tumor extracts were rendered nonreactive by heating at 50 or 56°C for 30 min, whereas the viral antigen was unaffected (Table 3). Secondly,

TABLE 3

EFFECT OF HEAT ON CF ANTIGEN TITERS OF TUMOR EXTRACTS AND VIRUS

Antigen	CF Antigen Titer versus Indicated Serum: Primary hamster tumor serum #5586 1:20 (8 antibody units)	Primary hamster tumor serum #2004 1:40 (≥4 antibody units)	SV40 immune monkey serum #2159 1:20	SV40 immune monkey serum #2159 1:80
1st passage transplant of primary SV40 hamster tumor, #2007				
Unheated	16	≥64	8	
Heated 56° 30′	0	0	0	
Transformed hamster cells, #3775*				
Unheated	32	32	8	
Heated 56° 30′	0	0	0	
Transformed hamster cells, #3797*				
Unheated	32		≥16	
Heated 50° 30′	(1)†		0	
Heated 56° 30′	0		0	
SV40 Virus				
Unheated	0	4		8
Heated 50° 30′	0	≥2		8
Heated 56° 30′	0	≥2		8

* #3775 and #3797 are tissue culture cell lines of SV40 transformed hamster embryo cultures (Melnick). See Table 2.

† Partial reaction undiluted.

the tumor antigens were chiefly "soluble," remaining unsedimented after centrifugation, whereas the viral antigen was completely sedimented (Table 4).

Characteristics of antibody responses to viral and tumor antigens in tumor-bearing hamsters: In a previous report,[10] we described the patterns of neutralizing and viral CF antibody responses in hamsters developing tumor after inoculation with virus when 2 days old. Tumor development in these animals was accompanied by development of rising titers of neutralizing antibody, the titer tending to parallel the size of tumor. CF antibody to viral antigen also developed, but usually much later and in lower titer than the neutralizing antibody.

One hundred and seven sera from 34 hamsters with primary SV40-induced tumors were tested for CF antibody reactive with tumor extracts. These sera are serial samples from the animals described in reference 10. The upper portion of Table 5 summarizes the relationship of this CF antibody to size of tumor at the time of bleeding. No antibody was detected prior to tumor development, while antibody appeared in moderate titer in the majority of hamsters with early tumors, and tended to rise in titer as tumors enlarged. In all, 31 of the 34 animals developed antitumor CF antibody.

The great majority of hamsters with tumors produced by transplantation of the THK-1 line[16] of *in vitro* transformed hamster kidney cells, or of the clones derived therefrom,[17] also developed antibodies to tumor extracts (Table 5, bottom), and in generally higher titers than in the animals with primary tumors.

Many of these serial serum specimens were also tested for CF and neutralizing antibody to SV40; the relationships of titers of antiviral and antitumor antibodies are shown in Table 6. Hamsters with primary tumors showed a slight correlation between tumor CF antibody and neutralizing or viral CF antibody titers, in that

TABLE 4

ULTRACENTRIFUGATION OF CF ANTIGENS IN TUMOR EXTRACTS AND VIRAL HARVEST

Material tested[c]	Primary Tumor #2007[a]		Transformed Cells #3775[b]		SV40 Virus (BSC-1 TC)	
	Primary hamster tumor serum #5586 1:10 (16 antibody units)	SV40 immune monkey serum #2159 1:20	Primary hamster tumor serum #5586 1:10 (16 antibody units)	SV40 immune monkey serum #2159 1:20	Primary hamster tumor serum #5586 1:10	SV40 immune monkey serum #2159 1:80
Starting suspension	16[d]	8	16	4	0	2
Centrifugal fractions						
Upper supernatant	8	2	8	4	0	0
Lower supernatant	16	4	16	8	0	0
Pellet (10×)	16	(AC)[e]	1	0	0	8

[a] This tumor extract was not reclarified prior to ultracentrifugation. Precipitates formed as a result of storage in the frozen state may have been responsible for the appearance of antigen in the pellet fraction.
[b] For description see Table 2.
[c] For centrifugation procedure see *Materials and Methods* section.
[d] CF antigen titer.
[e] The low titer reaction observed could not be evaluated because of anticomplementary activity at a 1:2 dilution.

TABLE 5

RELATIONSHIP OF "ANTITUMOR" CF ANTIBODY TITER TO SIZE OF TUMOR

Type of tumor	Size of tumor*	CF Antibody Titer to Tumor Antigen <5	5–20	40–>160	No. sera pos†: no. tested	
Primary	Pretumor	35‡	0	0	0/35	
	Small	5	11	5	18/23	(78%)
	Medium	8	10	14	29/37	(78%)
	Large	2	3	6	10/12	(83%)
Transplanted THK-1 cells§	Pretumor	11	0	0	0/11	
	Small	6	2	2	4/10	(40%)
	Medium	3	4	27	31/34	(91%)
	Large	1	0	8	8/9	(89%)

* Small = 0.1–1.0 cm mean tumor diameter. Medium = 1.1–3.5 cm mean tumor diameter. Large = >3.5 cm mean tumor diameter.
† Includes 8 sera which were positive in screening test but on which titers were not determined.
‡ Number of sera with indicated titer.
§ For description of THK-1, see Table 2.

animals with high titer tumor CF antibody tended to have the highest antiviral titers. In view of the correlation of all three titers with tumor size, the lack of clear correlation here is all the more striking. Tumors induced with transformed cells rarely induced antiviral antibodies; the viral CF and neutralizing antibodies which were detected were confined to sera of two animals with large tumors and high anti-tumor CF antibody. Neither of these two tumors contained demonstrable infectivity, as judged by tests of three serial biopsy specimens from one, and an autopsy specimen from the other. Although these data suggest some relationships between viral and tumor antigens, they also provide clear evidence that they are not identical.

Discussion.—The data presented here have established that cells which have become tumorous or which have undergone transformation subsequent to infection with SV40 consistently elaborate a specific CF antigen(s) not found in normal tissue or other tumors. The predominant antigen is clearly distinct from the SV40 antigen(s) detected in viral suspensions by the routine CF and neutralization procedures, both in serologic reactivity, as evidenced by the reactions with hamster antisera, and physically, as shown by the differences in thermal inactivation and sedimentation characteristics. However, present data do not exclude the possibility that this "tumor" antigen may be present in subdetectable amounts in viral harvests, and do not necessarily imply that its presence is restricted to transformed cells or cells with an integrated cell-virus relationship.

TABLE 6

COMPARISON OF ANTITUMOR AND ANTIVIRAL ANTIBODIES IN HAMSTER SERA

"Antitumor" CF antibody titer	Number of Sera with Titers in Range Indicated: Primary Tumors				Transformed Cell-Induced Tumors			
	Antiviral CF antibody titer							
	0	5–10	20–40	≥80	0	5–10	20–40	≥80
0	9	3	1	0	20	0	0	0
5–10	17	3	0	0	1	0	0	0
20–40	12	2	0	0	9	0	0	0
≥80	6	5	3	1	32	2*	1*	0
	Neutralizing antibody titer							
	0	5–10	20–40	≥80	0	5–10	20–40	≥80
0	1	3	4	0	12	2	0	0
5–10	5	6	0	0	0	0	0	0
20–40	2	7	1	0	9	0	0	0
≥80	0	0	4	4	23	1*	2*	3*

* In the tests of sera from hamsters with tumors induced with transformed cells, the 3 sera positive in CF and the 6 sera positive in neutralization tests were all from 2 animals.

Although the predominant antigen in tumor extracts is distinct from the presently known "viral" antigen(s), it is not clear whether the latter is also present in the neoplastic cells in immunologically significant amounts. It appears that SV40 tumors[18, 19] and transformed cells[16, 17] contain the viral genome in a highly integrated state, and may occasionally liberate small amounts of infectious virus. However, as judged by the relation between infectivity and CF antigen titers in AGMK harvests, the quantity of virus produced by tumor cells is far too small to be detected by the CF test, and is probably inadequate even to induce antibody response. Previous studies of antibody responses of hamsters with primary SV40-induced tumors suggested that significant amounts of viral antigens were indeed elaborated, since neutralizing antibody titers rose proportionally to size of tumor, even in animals with noninfectious tumors.[10] However, a few animals with large tumors failed to develop neutralizing or viral CF antibody. As reported here, tumors induced with transformed tissue culture cells rarely stimulated antiviral antibody response, suggesting that there may be a difference between primary and transformed cell-induced tumors with regard to content of viral antigen. This discrepancy may be due to differences in degree of expression of the viral genome, or to the booster effect of small amounts of viral antigen in primary tumors on the minimal antibody response produced by the initial virus inoculum. In this regard it is of interest that two hamsters with apparently noninfectious tumors induced by transplantation of transformed cells developed rising titers of neutralizing and viral CF antibody similar to the responses observed in primary tumors.

The consistent reactivity of hamster tumor and transformed cell suspensions with the SV40 immune monkey serum could also indicate presence of viral CF antigens. However, the reacting antigen showed the heat instability and low sedimentation rate which were characteristic of the "tumor-specific" antigen; also, the quantitative aspects of the reactivity differed markedly from the reaction of this serum with viral antigen. It seems more likely that the monkey serum contains two distinct types of antibody, one against virus and one against "tumor-specific" antigen formed during the course of a subclinical and presumably nononcogenic infection.

The relationship of the new cellular antigen described here to that responsible for rejection of SV40 tumor transplants by hamsters immunized with SV40[7–9] is

not known. The only available evidence suggests that they are distinct, since animals hyperimmunized with SV40 did not develop CF antibody to tumor antigens (Table 1) and these animals presumably would be resistant to tumor challenge.

The presence of the tumor CF antigen in all species transformed cells tested provides strong evidence that it is formed from information present in the viral genome. The alternative, that the information for formation of the antigen is of host cell origin, made functional by the viral infection, seems incompatible with the specificity of the antigen and with its presence in five genera of mammals. The presence of the antigen, coupled with the difficulty of detection of infectious virus in transformed rabbit and mouse cells,[11] suggests that the genetic information of SV40 is present in these cells in an integrated state similar to that in hamster cells. It is noteworthy that this apparently integrated virus-cell relationship has not led to overtly malignant cell behavior in the case of the C3H mouse cells, which do not transplant in isologous hosts;[11] also, in the case of the bovine cell cultures, production of the antigen was not accompanied by noticeable alteration in cellular morphology or growth pattern.

The findings reported herein are similar in many respects to the findings of Heubner *et al.*[6] in virus-free hamster tumors induced by adenovirus types 12 and 18; however, in the adenovirus tumors, noninfectious viral antigens are easily demonstrated. There is also evidence for tumor specific antigens not found in viral harvests, but their identification as wholly new antigens is not clearly resolved.

Summary.—Suspensions of hamster tumors and various species cells transformed by SV40 contain a specific antigen(s) demonstrable by CF tests with sera of hamsters bearing SV40 tumors. This antigen has several properties which are distinct from the SV40 viral antigen(s); it is soluble, heat labile, and has a different serologic reactivity. These results, taken together with other evidence, support the hypothesis that the new antigen is synthesized by information from SV40 viral genome integrated in the tumor and *in vitro* transformed cells.

Antibody to the "new" cellular antigen appears in the serum of hamsters soon after the development of tumor, and rises in titer as the tumors increase in size. Response to this antigen is largely independent of antiviral antibody response.

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VIRAL INHIBITION IN THE METAPHASE-ARREST CELL

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RNA-virus development proceeds normally in cells specifically inhibited for DNA or RNA synthesis.[1-7] Thus, in cells exposed to inhibitors of DNA or DNA-dependent RNA synthesis, Mengovirus,[2-4] poliovirus,[5, 6] and Newcastle disease virus[1, 7] show characteristic growth curves and essentially normal yields. During mitosis, cellular RNA is not synthesized.[8, 9] The natural suppression of RNA synthesis from late prophase to early telophase provides a system to examine viral replication in a cell devoid of DNA and RNA synthesis. It permits inquiry into the question of whether normal regulatory processes of the cell can exert control over an invading viral nucleic acid. To explore this problem we have used large populations of cells arrested in metaphase. Primarily this study describes the inhibition of viral replication following the interaction of metaphase-arrest HeLa cells with an RNA virus of the Myxovirus family, Newcastle disease virus (NDV). Experiments reported here also demonstrate inhibition of a small RNA virus (poliovirus) and a DNA virus (vaccinia), under similar conditions, and provide evidence for viral inhibition in the normal metaphase cell. Actinomycin D-treated and metaphase-arrest cells are compared as hosts for viral replication.

Materials and Methods.—Preparation of metaphase-arrest cells: Clonal line HeLa S3 cells were grown as monolayers in attachment solution[10] minus Ca^{++} or Eagle's spinner medium[11] in which Na_2HPO_4 was replaced with NaCl, and calf serum added to a final concentration of 6%. Cells were arrested in metaphase using the spindle inhibitor, vinblastine sulfate (Velban, Lilly) added at 0.05 to 0.1 μg/ml of medium. Cells in metaphase-arrest were harvested from the monolayer preferentially by employing a shaking technique developed by Terasima and Tolmach.[12] Omission of Ca^{++} from the growth medium greatly facilitated this procedure by producing a tenuously attached metaphase-arrest cell. The populations were concentrated by centrifugation and resuspended in appropriate medium. It was possible to obtain readily over 10^8 cells of which 98–99% were in metaphase-arrest. Vinblastine-exposed cells not recruited into metaphase-arrest, i.e., those remaining attached to the glass substrate following harvesting of the arrested cells were detached by trypsinization and used as control-interphase cells.

Virus-cell interaction: Monodisperse HeLa cells were exposed to NDV-strain Calif. by means of the dual temperature-dilution sequence already described.[13] Virus attachment was effected at 2°C while dilution, virus penetration, eclipse, and growth were carried out at 37°C. This procedure imparts a relatively high degree of synchrony to the initiation of infection. Assessment of virus multiplicity was by plaque assay on chick embryo cell monolayers.[13] Infected cells were scored

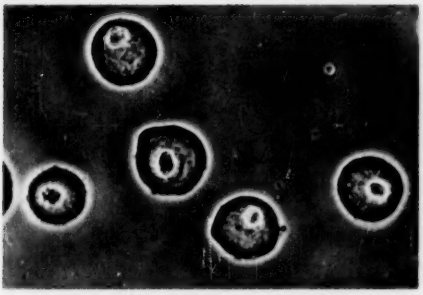

FIG. 1.—*HeLa cells in metaphase-arrest:* A phase-contrast photomicrograph of slightly squashed neutral red stained HeLa cells harvested after a $22^1/_2$-hr exposure to 0.1 μg vinblastine sulfate/ml of growth medium. Note the characteristic clumped chromatin and the absence of a defined membrane enclosing the chromatin mass.

by the single cell hemadsorption technique where individual cells specifically adsorb erythrocytes to a degree and in a pattern which reflects the stage of the virus growth cycle.[14]

H^3-uridine incorporation: A minor modification of the coverslip technique of Baltimore and Franklin[15] was used to study H^3-uridine incorporation. Coverslips of 22 mm diameter were placed atop no. 00 silicon rubber stoppers and inoculated with equal numbers of virus-cell complexes that had been washed and resuspended in cold attachment solution containing H^3-uridine (0.25 μC/ml) and only 0.06% calf serum. The cell density was 2.0×10^5 cells/0.25 ml/coverslip. Both interphase and metaphase-arrest cells attached to the coverslip firmly enough to permit removal of the supernatant fluid. The attached cells were dried on a hot plate at 80°C, fixed in cold acetic-ethanol (1:2) for 10 min, rinsed in cold 70% ethanol, cold water, and finally extracted with 2% cold perchloric acid for 1 hr. Coverslips were rinsed copiously with a large volume of water, dried on a hot plate, and β-particle activity determined in a windowless, gas-flow counter. Autoradiography was carried out using the technique of Franklin and Rosner.[16]

Development of metaphase-arrest populations: Large populations of metaphase-arrest cells were routinely prepared by the selective harvesting of the tenuously attached metaphase cell as described above. In a representative experiment a monolayer of 4.0×10^7 HeLa cells, exposed to vinblastine sulfate at 0.1 μg/ml for 20 hr in low Ca^{++} medium and shaken to dislodge metaphase-arrest cells, was found to yield 1.9×10^7 cells in the supernatant fluid. These cells were concentrated by centrifugation and aliquots examined with phase-contrast microscopy. Metaphase-arrest cells were identified by characteristic clumping of chromatin in a single mass, and the absence of a membrane enclosing the nucleus (Fig. 1). Interphase cells were distinguished by clearly defined nuclei containing one or more nucleoli. In a sample counting, metaphase-arrest cells numbered 687 and interphase cells 12, i.e., 98.2% of the population was in metaphase-arrest. Cells which remained on the glass after shaking were removed with trypsin and found to total 1.8×10^7; over 98% of these cells were in interphase. A small fraction of cells, <10%, disintegrated or showed gross cytopathology, possibly as a result of vinblastine toxicity.

Metaphase-arrest populations are not indefinitely stable; however, it is possible to maintain them for about 30 hr when they are exposed continuously to the spindle inhibitor. Beyond this time a significant fraction of the cells shows cytopathic effects such as extensive blebbing and disintegration. If vinblastine is removed from the medium bathing the metaphase-arrest cells, a delay occurs before the onset of cytopathology, and a significant fraction of cells escapes the metaphase block and goes on to divide. For example, when vinblastine was removed after 20 hr, 36% of the metaphase-arrest cells divided within the next 13 hr. Only rarely will these dividing cells undergo another division; some form giant cells.

Results.—Hemadsorption studies: When HeLa cell monolayers were exposed to vinblastine sulfate for 20 hr, infected with NDV, and then incubated for 10 hr in the presence of the spindle inhibitor, the results presented in Table 1 were obtained: 94.8% of the interphase cells were hemadsorption positive (HAD^+)—proof that they were infected, while only 4% of the metaphase-arrest population maintained in the presence of the inhibitor were positive. Almost one half of this 4% represented contaminating interphase cells. Presumably the remaining HAD^+

TABLE 1

Cell population	Cells in interphase or metaphase-arrest at time of infection (%)		HAD⁺ and HAD⁻ cells 10 hr postinfection (%)			
			+ Vinblastine*		− Vinblastine	
	Interphase	Metaphase-arrest	HAD⁺	HAD⁻	HAD⁺	HAD⁻
Interphase	96.5	3.5	94.8	5.2	95.4	4.6
Metaphase-arrest	1.8	98.2	4.0	96.0	28.0	72.0

* Presence of vinblastine sulfate at 0.1 μg/ml in attachment solution indicated by +, and absence by −.

cells were recruited into metaphase *following* infection. Thus, it would appear that very few, if any, metaphase-arrest cells produce viral hemagglutinin.

In the absence of inhibitor, infected metaphase-arrest populations showed a significant increase in HAD⁺ cells, i.e., 28% compared to the 4% in the presence of the inhibitor. As shown below, this increase is due to "escape" of many cells from the metaphase block and a subsequent initiation of virus production.

Figure 2 shows a representative infected metaphase-arrest and interphase cell. The metaphase-arrest cell is HAD⁻ while the interphase cell, *exposed for the same time* to the vinblastine sulfate, is HAD⁺.

Virus attachment: Perhaps metaphase-arrest cells were not infected because their surface was altered so as to prevent virus attachment. Data presented in Figure 3 demonstrate that the same kinetics govern virus attachment to both metaphase-arrest and interphase cells and thus rule out this possibility.

Penetration, eclipse, and infectious virus formation: Steps subsequent to virus attachment were examined in infected cells as follows: suspensions of appropriate cells were exposed to NDV by the dual temperature-dilution sequence; surface-situated virus was neutralized with specific antibody, excess antibody washed away, and the virus-cell complexes plated on chick embryo cell monolayers for plaque formation. It is important to record that metaphase-arrest cells do not attach to chick embryo cell monolayers in the standard plaquing procedure; therefore, all virus-cell complexes were plated in a very thin agar overlay (1 ml/60 mm dish) which when hardened was in turn overlayed in the usual fashion. Under the conditions indicated in Table 2, both normal and vinblastine exposed-interphase cells infected with NDV and plated in the *absence* of vinblastine produced the maximum plaque count observed. In contrast, arrested cells did not function as infective centers. Thus, all plaque-yielding virus-cell complexes in the metaphase-arrest population, scored by plating in the *absence* of vinblastine (24%, in Table 2), can be accounted for by the fraction of cells that escape

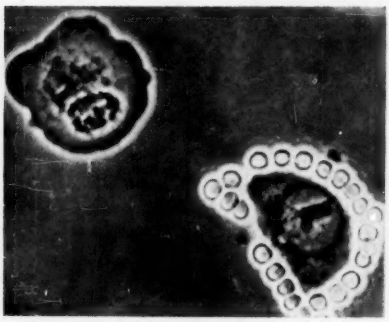

FIG. 2.—*Comparison of an HAD⁺ interphase and HAD⁻ metaphase-arrest cell:* A high magnification phase-contrast photomicrograph of an interphase and metaphase-arrest cell from a population of cells infected with NDV (m = 2.7) and tested for hemadsorption with bovine erythrocytes 10 hr later. The nucleus and nucleoli are clearly visible in the HAD⁺ interphase cell that shows a circumferential HAD pattern. Clumped chromatin is evident in the HAD⁻ metaphase-arrest cell. The bovine RBC has a diameter of approximately 5 μ.

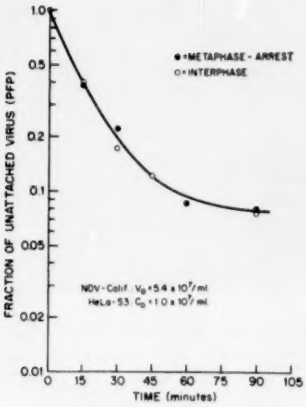

FIG. 3.—*Kinetics of NDV attachment to interphase and metaphase-arrest HeLa cells:* Equal numbers of NDV plaque-forming particles (PFP) were added to suspensions of interphase and metaphase-arrest cells at 2°C. The metaphase-arrest population contained 98.3% arrested cells. Aliquots of the virus-cell mixture were removed at various intervals, centrifuged, and the supernatant fluid assayed for unattached virus. Note that the attachment curves are the same for both cell types.

metaphase-arrest. This conclusion is substantiated further by the demonstration of essentially complete suppression of plaque formation from infected metaphase-arrest cells plaqued in the presence of vinblastine, i.e., under conditions where escape from the metaphase block does not take place (Table 2). Therefore, we conclude that metaphase-arrest cells infected with NDV do not produce infectious virus as long as the host cell is maintained in metaphase-arrest. In the absence of vinblastine some infected cells escape the metaphase block and are able to initiate new virus production. The last column in Table 2 demonstrates that besides displaying normal attachment kinetics on the metaphase-arrest cell, NDV also enters a true eclipse state. This follows from the fact that over 90 per cent of the plaquing centers are destroyed when interphase or metaphase arrest-virus complexes are freeze-thawed 3 times.

TABLE 2

Cell population	Per cent of Maximum Plaque Count		
	NDV-HeLa*	NDV-HeLa (vinblastine in overlay)	NDV-HeLa (3× freeze-thaw)
Interphase	100	57	7
Metaphase-arrest†	24	2.2	1.5

* NDV-HeLa complexes at $m \doteq 2.9$ plated on chick embryo cell monolayers.
† 98.2% of HeLa cells in metaphase-arrest.

These results do not appear restricted solely to the HeLa cell as host. When vinblastine sulfate was added to chick embryo cell monolayers to assess its effect on NDV plaque formation, an unusual phenomenon was observed; many of the chick embryo cells became arrested in metaphase and survived destruction from the high concentration of virus that accumulates in a plaque. Thus, the nonstaining plaque areas were studded with many neutral red-vitally stained cells arrested in metaphase.

Inhibition of viral-induced RNA synthesis: Tests for viral-induced RNA synthesis in metaphase-arrest cells were prefaced by the demonstration that such synthesis could be detected in NDV-infected HeLa cells, especially since yields of infectious virus in this system are notoriously low.[1, 18, 19] Combining the procedure used by Kingsbury for demonstrating NDV-induced synthesis in actinomycin D-treated chick embryo cells,[7] and the coverslip technique of Baltimore and Franklin,[15] we were able to detect a significant level of acid insoluble H^3- uridine incorporation into the NDV-infected HeLa cell. Actinomycin D (AcD) at 5 μg/ml and H^3- uridine at 0.25 μC/ml were added to the virus-cell complexes once virus

TABLE 3

	Cell population	Treatment	Virus	CPM*
(*A*)	Interphase	—	—	6110 ± 517
	Interphase	Actinomycin D	—	183 ± 10
	Interphase	Actinomycin D	NDV†	1692 ± 220
(*B*)	Metaphase-arrest‡	Vinblastine	—	131 ± 26
	Metaphase-arrest	Vinblastine	NDV	126 ± 21

* H^3-uridine incorporated as acid insoluble cpm/2.0 × 10^5 HeLa cells/coverslip for 13 hr.
† Newcastle disease virus: m = 3.6. Time = 13 hr postinfection.
‡ 97.5% HeLa cells in metaphase-arrest.

TABLE 4

	Cell population	Treatment	Virus	CPM[a]
(*A*)	Interphase[b]	—	—	7133 ± 665
	Interphase	Actinomycin D	—	105 ± 2
	Interphase	Actinomycin D	Polio[c]	1793 ± 250
(*B*)	Metaphase-arrest[d]	Vinblastine	—	302 ± 47[e]
	Metaphase-arrest	Vinblastine	Polio	321 ± 56

[a] H^3-uridine incorporated as acid insoluble cpm/2.0 × 10^5 HeLa cells/coverslip for 6 hr.
[b] Vinblastine-exposed interphase cells harvested by trypsinization after 17 hr.
[c] Multiplicity of attached poliovirus about 4 PFP/cell.
[d] 97.8 per cent cells in metaphase-arrest.
[e] These values are not corrected for the contribution of contaminating interphase cells.

penetration at 37°C was completed (15 min), and maintained in the medium for the duration of the experiment—usually 10–13 hr. Table 3 presents the mean and standard deviation of acid insoluble counts obtained from coverslip platings run in quadruplicate. Data in Table 3 (*A*) demonstrate the well-documented reduction in cellular DNA-dependent RNA synthesis in the presence of AcD, and a 9-fold increase in label incorporation into the virus-infected AcD-treated interphase cell after a 13-hr exposure to H^3-uridine. Under these same conditions *vinblastine*-exposed interphase cells behave similarly. Metaphase-arrest cells were substituted for AcD-treated interphase cells, and the experiment was repeated. Results in Table 3 (*B*) demonstrate that (1) metaphase-arrest cells manifest an extremely low level of acid insoluble H^3-uridine incorporation,[20] and (2) RNA synthesis is *not* induced in metaphase-arrest cells which attach NDV under conditions adequate to support a marked degree of viral induced synthesis in AcD-treated interphase cells. Further analyses of the AcD-treated cells demonstrated that equal fractions of interphase or AcD-treated interphase cells were HAD^+ at the end of the 13-hr incubation period, additional evidence that *all* NDV-infected AcD-treated HeLa cells synthesize viral hemagglutinin.

Inhibition of poliovirus-induced RNA synthesis in the metaphase-arrest cell: Certain aspects of the experiments reported above were extended to include a member of the picornaviruses, i.e. poliovirus, known to suppress markedly host-RNA synthesis and to remain virtually unaffected by AcD treatment. Poliovirus-induced RNA synthesis was measured by the techniques described above for NDV. Data in Table 4 (*A*) demonstrate poliovirus-induced RNA synthesis in AcD-treated HeLa cells. These cells support a 17-fold increase in acid insoluble H^3-uridine incorporation over a 6-hr incubation period compared to uninfected AcD-treated controls. Table 4 (*B*) shows that the same multiplicity of poliovirus, about 4 PFP, does *not* induce RNA synthesis in the metaphase-arrest cell. However, preliminary experiments using much higher multiplicities of virus reveal up to a 4-fold increase in the level of H^3- uridine incorporation. We have deter-

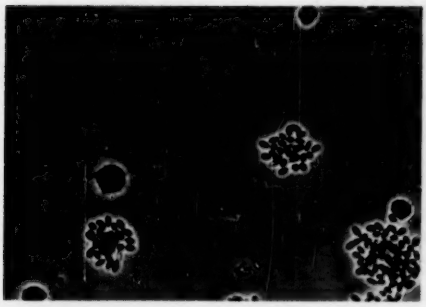

FIG. 4.—*Hemadsorption tests on vaccinia virus-infected interphase and metaphase-arrest HeLa cells:* Turkey erythrocytes are seen bound to vinblastine exposed-interphase HeLa cells infected $10^1/_2$ hr previously with vaccinia virus. The interphase cells, recognized by their spread configuration, are HAD^+. The population consisted of 30% metaphase-arrest cells. These cells, characterized by their spherical appearance and clumped chromatin (not visible here), are all HAD^-.

mined from autoradiographic analyses that this increase represents about 20% of the metaphase-arrest population incorporating label at a high level.

Vaccinia virus interaction with the metaphase-arrest cell: Observations reported above were extended in part to a DNA virus. Synthesis of a specific hemagglutinin of vaccinia virus was scored by a modification of the single cell hemadsorption technique.[14] Monolayers of HeLa cells which contained metaphase-arrest cells, and a series of untreated cells, were exposed to vaccinia virus for 1 hr at 37°C, washed free of unattached virus, and incubated in attachment solution without vinblastine. Hemadsorption tests were run $10^1/_2$ hr later and the fraction of HAD^+ and HAD^- cells compared in the two series. Vaccinia virus-treated HAD^- metaphase-arrest cells were scored readily since they stood out in sharp contrast to the HAD^+ interphase cells (Fig. 4). The results presented in Table 5 demonstrate the inhibition of viral hemagglutinin accumulation on the surface of the vaccinia infected metaphase-arrest cell. There were over 70 times more HAD^- cells in the metaphase-arrest population than in the interphase population. Furthermore, during the $10^1/_2$-hr postinfection period approximately 30% of the cells in metaphase-arrest appear to have escaped the metaphase block. These cells apparently became HAD^+ since no HAD^- flattened cells, i.e., interphase, or metaphase-arrest escapees, were observed in the several hundred scored.

TABLE 5

Cell population	Number of Cells HAD^+*	Number of Cells HAD^-	Fraction of HAD^- cells
Interphase	669	2	0.003
Metaphase-arrest†	612	164	0.211

* Hemadsorption test run $10^1/_2$ hr postinfection. Incubation was in the absence of vinblastine.
† 31% cells in metaphase-arrest at time of infection.

Virus interaction with the normal mitotic cell: The data presented thus far strongly suggest that viral inhibition is not a direct consequence of drug action but a reflection of the biochemical-morphological state of the cell once recruited into metaphase-arrest. The validity of these conclusions was borne out in experiments presented below which employed the *normal* mitotic cell as host.

The brief interval spanning late prophase to early telophase in the HeLa S3 cell (*ca.* 30 min.[17]) precludes viral life cycle analysis. However, the following considerations lead to an experimental approach which circumvented this shortcoming. If it is assumed that synthesis of viral RNA is not initiated during mitosis, and if a significant fraction of the virus latent period is concerned with initiating synthesis of RNA, then normal cells infected with NDV approximately an hour before mitosis would be expected to show an extension of the latent period at least equivalent to the time interval between metaphase and early telophase.

This effect would result in a disproportionately larger fraction of HAD$^-$ cells among a metaphase population harvested shortly after infection. The single cell hemadsorption technique provides the sensitivity required to resolve such an effect. The results of the experiment detailed in Table 6 suggest that some event critical to completion of the virus latent period does not occur during normal metaphase but is initiated once mitosis is completed.

TABLE 6

COMPARISON OF HEMADSORPTION IN HELA CELLS INFECTED WITH NDV PRIOR TO METAPHASE

Cell population*	Fraction of Cells HAD$^+$	HAD$^-$	Ratio: HAD$^-$/HAD$^+$
Interphase (1-cell colonies)	0.51	0.49	0.96
1½ hr pre-metaphase (2-cell colonies)	0.19	0.81	4.3

The following experiment was carried out entirely at 37°C. Monolayers of HeLa cells were exposed to NDV for 30 min to receive an attached multiplicity of about 5–10 plaque-forming particles, flooded with attachment solution, and 1½ hr later subjected to the selective harvesting procedure for the removal of cells in normal metaphase. Virus-cell complexes were plated immediately into 35 mm plastic Petri dishes, incubated, and after 1 hr for cell attachment, tested in duplicate at 20-min intervals for the appearance of HAD$^+$ cells. Interphase cells dislodged during the shaking procedure served as controls. Data were analyzed by dividing the cells into two classes, i.e., cells in (1) interphase or (2) metaphase *at the time of harvest*. Metaphase cells produced 2-cell colonies 1 hr after harvest and therefore could be readily distinguished from single interphase cells inadvertently harvested at the same time. When tested 2½ hr after infection, i.e., 1 hr after selective harvesting, the 2-cell class contained 4.3 times the frequency of HAD$^-$ colonies as observed in the 1-cell or interphase class. By 10 hr postinfection, this difference disappeared and both classes of cells contained equal fractions of HAD$^+$ cells.

* 58% and 42% of the cells were present as 1- and 2-cell colonies, respectively, when sampled for hemadsorption 1 hr post-plating, i.e., 2½ hr postinfection. Over 200 cells of each class were scored.

Discussion.—These data demonstrate that HeLa cells maintained in metaphase-arrest and infected with one or a few infectious particles of Newcastle disease virus do not function as infectious centers. Failure to achieve infection was not attributable to lack of cellular receptors since the kinetics of virus attachment to interphase and metaphase-arrest cells was identical. Virus penetration was adjudged normal in the metaphase-arrest cell since the addition of specific anti-NDV serum after a period of time ample to assure penetration of NDV in interphase HeLa (<15 min at 37°C) did not prevent infectious center formation by arrested cells that subsequently escaped the metaphase block. Newcastle disease virus eclipses normally in the metaphase-arrest cell as indicated by the 10-fold decrease in titer of infectious centers upon freeze-thawing under conditions optimal for detection of noneclipsed, cell associated virus.

The separate experiments that established essentially identical patterns of virus *attachment*, *penetration*, and *eclipse* in the metaphase-arrest and interphase HeLa cell gained independent confirmation from the fact that metaphase-arrest cells exposed to virus could ultimately initiate a fruitful cycle of NDV growth if they escaped the metaphase block subsequent to removal of vinblastine. Initiation of viral synthesis in cells escaping metaphase-arrest provides definite evidence that eclipsed virus, or some later stage of viral RNA-cell interaction, is stable within the cell for at least several hours.

Inhibition of NDV, vaccinia, and poliovirus replication was demonstrated in metaphase-arrest cells infected at low virus multiplicities. Multiplicities 10 to 20 times higher than reported here failed to reveal synthesis of NDV-induced hemagglutinin although a slight increase in H^3-uridine incorporation was noted. The situation with poliovirus was different: autoradiographic studies showed that at similarly high multiplicities a significant fraction of infected metaphase-arrest cells incorporated label at a high level. A subsequent report will consider what attri-

butes of high multiplicity infection with poliovirus conspire to induce ribonucleoside incorporation in the arrested cell.

The apparent hiatus in virus development during normal mitosis as well as in the metaphase-arrest cell make it appear likely that the phase of the growth cycle of the *host* when infected may influence the scheduling of events in the *viral* growth cycle. Thus, the length of the latent period may be expected to mirror the duration of mitosis as influenced by inherent or environmental factors, and perhaps contribute to the phenomenon of asynchrony of infection.[21]

The very early stages of viral invasion—i.e. attachment, penetration, and disappearance of infectivity—take place with equal facility in the metaphase-arrest and AcD-treated cell; this means that some subsequent critical event does not occur in the metaphase-arrest cell. The most immediate event in logical sequence is mobilization of viral-RNA from the nucleocapsid. Once mobilized, free viral-RNA must utilize the protein synthesizing system to transcribe the first protein programmed by viral messenger. Experiments are in progress to determine whether failure to (1) mobilize viral-RNA, (2) initiate polyribosome formation, or (3) initiate or sustain some subsequent event in viral replication, accounts for viral inhibition in the mitosing cell.

Summary.—Infectious Newcastle disease virus formation, viral hemagglutinin production, and viral-induced RNA synthesis are inhibited in HeLa cells arrested in metaphase. Under identical conditions of infection, high levels of viral synthetic activity are demonstrable in actinomycin D-treated cells. Virus attachment, penetration, and eclipse proceed normally in the metaphase-arrest cell. Viral replication is self-initiating in cells that escape the metaphase block. NDV development is apparently suppressed for the duration of *normal* mitosis.

Vaccinia-induced hemadsorption and poliovirus-induced RNA synthesis are inhibited in the metaphase-arrest cell exposed to virus multiplicities sufficiently high to infect 95–99% of the cells, and in the case of poliovirus, to induce a 17-fold increase in RNA synthesis in actinomycin D-treated HeLa.

This investigation was aided by grants AI-03619-03 and GM-07348-03 from the National Institutes of Health. Dr. Marcus is a Research Career Development Awardee of the National Institute of Allergy and Infectious Diseases, U. S. Public Health Service (GM-K3-15,461-C2-A). Preliminary results reported in *Fed. Proc.* (Abst.), **22,** 645 (1963).

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*THE DNA CONTENT OF THE CHLOROPLASTS OF ACETABULARIA**

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On the basis of microscopic observations and genetic analysis, it is generally believed that subcellular organelles such as plastids are self-duplicating bodies, and they are endowed with a degree of genetic autonomy.[1]

With the demonstration of the central role of the nucleic acids in the biological transfer of information, it became important to determine whether the genetic properties associated with these organelles are also attributable to nucleic acids within the organelles.

Reports have appeared based on either histochemical and electron-microscopical evidence or analysis of isolated chloroplasts which suggest the presence of DNA in the chloroplasts.

Stocking and Gifford[2] demonstrated the incorporation of tritium-labeled thymidine into the chloroplasts of Spirogyra. Sagan and Scher[3] similarly showed such incorporation into the plastids of Euglena and, furthermore, the labeled molecules disappeared after DNAase digestion. Ris and Plaut[4] found Feulgen-positive structures in the chloroplasts of Chlamydomonas; DNAase treatment caused the disappearance of these structures. By electron-microscopy they found fibrillar material in corresponding regions of the chloroplasts; the fibrils were absent in DNAase-treated samples. Fibrils with similar properties were also found in etiolated plastids of higher plants.[5]

These observations are convincing that DNA is indeed present in the plastids. However, the quantitative estimations of such DNA which rely on the analysis of plastids isolated from homogenized cells are less convincing. For example, DNA values of up to 0.5 per cent of the total dry weight of isolated chloroplasts were reported by Biggins and Park.[6] A fundamental objection to data derived from isolated chloroplasts is that the DNA may have arisen from nuclear contamination. The gravity of this objection is illustrated in the recent report by Chun *et al.*[7] on the fractionation by density-gradient ultracentrifugation of the DNA derived from

green plants. DNA which was isolated from whole cells was found to be composed of a major and minor component. The major component, "α," presumably derived from the nucleus, comprised about 99 per cent of the total DNA; the minor 1 per cent, "β," was presumed to be derived from the plastids. DNA which was extracted from "purified" chloroplast fractions was also found to be composed of the two fractions. The "β" fraction now amounted to 20–40 per cent of the DNA, but the nuclear, "α" fraction still comprised at least 60 per cent of the total DNA.

We sought, therefore, to make further quantitative determinations of the DNA in chloroplasts. To overcome the objection of the contamination of the chloroplast preparations with broken nuclei, we decided to use as a source of chloroplasts the giant unicellular alga, Acetabularia. This large unicellular organism contains a single nucleus which is located at the basal portion of the cell.[8] Enucleation can be achieved easily by cutting off the base of the cell.

Another source of contamination with DNA is from bacteria which normally grow adsorbed to the algal cell wall. It was important, therefore, to grow this alga in bacteria-free cultures and isolate the chloroplasts only from enucleated segments of the cells.

In this paper, we describe the aseptic culturing of Acetabularia, the isolation of chloroplasts from them, and the determination of the DNA in these chloroplasts by a fluorometric procedure for deoxypentose before and after DNAase digestion.

Methods and Materials.—Acetabularia cultures: Acetabularia mediterranea cells were obtained through the generosity of Dr. J. Brachet from his stock cultures. We are grateful to him for this gift. To obtain cultures of this alga, free from bacteria, we treated the plants as follows: mature intact plants were immersed for 1–2 hr in a 10% solution of silver-protein "Argyrol" (Crooks-Barnes Lab., Inc., Wayne, New Jersey) in sea water. The cells were then washed thoroughly with autoclaved, sterile sea water and then immersed for 5 days in a solution of antibiotics in sea water. The antibiotic solution was of the following composition: streptomycin sulfate, 200 mg; penicillin, 100 mg; mycostatin, 20,000 units; chloramphenicol, 20 mg; neomycin, 20 mg; sea water, 100 ml. This solution was passed through a Millipore filter before use. After the antibiotic treatment, the cells were washed with sterile sea water, and then the caps were removed, cut, and the cysts collected aseptically. (This treatment for obtaining bacteria-free cultures may also be used on young, growing Acetabularia cells as well as on several multicellular green algae.)

The germination of the cysts and further cultivation procedures were based on the methods of Hammerling.[9] The cysts were washed several times with sterile sea water, then for about 3 min in sterile distilled water to initiate germination, and transferred to an Erlenmeyer flask containing sea water. The flasks with cysts were illuminated with 500 ft-c of white light for 16 hr each day. Swimming gametes appeared within a week; they were immediately transferred into flasks with culture medium and put in the dark where they settled evenly on the bottom of the flask. After 24 hr the flasks were returned to light. If not transferred immediately, the zygotes adhered in thick clusters to the illuminated side of the flask. Such zygotes had to be dislodged with a sterile needle, and after shaking well to disrupt the clusters of cells, they were transplanted into flasks containing culture medium.

The culture medium was of the following composition: 900 ml sea water + 100 ml distilled water + 10 ml soil extract[10] + 75 mg KNO_3. This solution was sterilized by autoclaving. After the solution cooled, it was further enriched with sterile K_2HPO_4, 15 mg; glucose, 500 mg; and tryptose (Difco), 500 mg. The pH was adjusted to 7.5 with sterile K_2CO_3. The organic enrichments served to accelerate the growth of chance contaminants of the cultures. Flasks which became turbid due to contamination were discarded.

The cultures were maintained at 23°C under a bank of white fluorescent lights with illumination of approximately 500 ft-c. The light was kept on for 16 hr daily.

The cultures were grown in cotton-plugged, 1-liter Erlenmeyer flasks, containing about 400 ml culture medium. Satisfactory growth was obtained without changing the medium if the plants

were not overcrowded; about 100 cells per flask was satisfactory. However, changing the medium once a month gave a rapid growth of cells in flasks containing several hundred cells.

Chloroplast isolation: Cells were harvested for analysis when they had grown to about 1–2 cm in length and before the beginning of cap formation. The cells were enucleated by cutting off the basal $^1/_3$ portion of the cell. The enucleated portions were collected in sterile sea water and washed with several changes of sea water. About 1,000 cells were used for each experiment. All further operations were done in the cold at approximately 5°C. For grinding, the enucleated segments were transferred by forceps to a homogenizer with a loose-fitting pestle (Potter-Elvehjem type). After draining off all excess sea water, the cell fragments were washed once with a sucrose medium of the following composition: 0.3 *M* sucrose in 0.03 *M* phosphate buffer, pH 7.4, and 0.001 *M* EDTA. The cell fragments were ground with approximately 3 times their volume of this medium. The grinding was done gently by hand to avoid excessive breakage of the plastids. The ground material was diluted with the sucrose medium, and this green extract was layered over a 0.5-*M* sucrose solution in centrifuge tubes. The tubes were centrifuged for 5 min at approximately 100 *g*. This caused the sedimentation of the broken wall material and other heavy cell debris into the lower layer. The green, upper layer was transferred to a second tube and centrifuged for 15 min at 1,500 *g*. The supernatant solution was discarded, and the sediment resuspended in the 0.3-*M* buffered sucrose solution. This differential centrifugation was repeated once more. The green sediment, after the second 15-min centrifugation at 1,500 *g*, was suspended in a small volume of the sucrose solution, and samples taken for analysis. Microscopic observation under phase indicated that this green suspension was composed primarily of intact chloroplasts with few smaller nongreen structures.

Analytical methods: DNA analysis was performed by a fluorometric procedure based on the procedure of Kissane and Robins[11] and modified by Izawa *et al.*[12] to include a DNAase digestion step in the preparation of the samples. Reagent blanks and DNA standards (highly polymerized DNA, Worthington) were run with each set of determinations. The DNAase used was obtained from the Worthington Company.

Chlorophylls were determined as the pheophytins by the method of Vernon.[13] Protein N was determined by a micro-Kjeldahl procedure. A flow diagram for the analytical procedures used is described in Figure 1.

Results.—Typical results of DNA analyses on DNA-enriched fractions which were isolated from chloroplasts, as shown in Figure 1, are presented in Table 1.

Net DNA may be estimated by subtracting the value of fraction 4, the presumed "DNA" which was solubilized by buffer only, from fraction 2, the DNA which was solubilized by DNAase action; also, by subtracting fraction 1, the

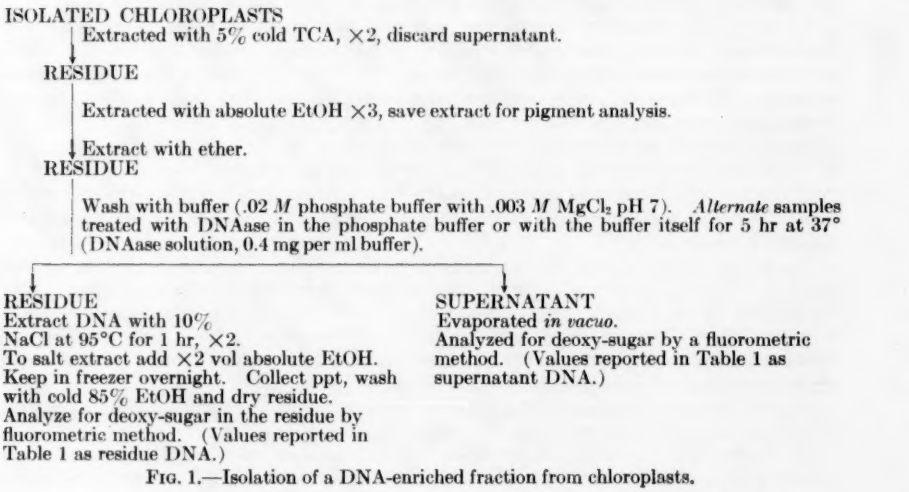
ISOLATED CHLOROPLASTS
Extracted with 5% cold TCA, ×2, discard supernatant.
RESIDUE
Extracted with absolute EtOH ×3, save extract for pigment analysis.
Extract with ether.
RESIDUE
Wash with buffer (.02 M phosphate buffer with .003 M MgCl2 pH 7). Alternate samples treated with DNAase in the phosphate buffer or with the buffer itself for 5 hr at 37° (DNAase solution, 0.4 mg per ml buffer).
RESIDUE
Extract DNA with 10% NaCl at 95°C for 1 hr, ×2.
To salt extract add ×2 vol absolute EtOH. Keep in freezer overnight. Collect ppt, wash with cold 85% EtOH and dry residue.
Analyze for deoxy-sugar in the residue by fluorometric method. (Values reported in Table 1 as residue DNA.)
SUPERNATANT
Evaporated in vacuo.
Analyzed for deoxy-sugar by a fluorometric method. (Values reported in Table 1 as supernatant DNA.)

FIG. 1.—Isolation of a DNA-enriched fraction from chloroplasts.

TABLE 1

DETERMINATION OF DNA IN THE DNA-ENRICHED FRACTIONS FROM ACETABULARIA CHLOROPLASTS

	Fraction	DNA-determined mμg
A*		
Samples treated with DNAase	1. Residue	5
	2. Supernatant	90
Samples treated with buffer only	3. Residue	80
	4. Supernatant	30
B†		
Samples treated with DNAase	1. Residue	5
	2. Supernatant	85
Samples treated with buffer only	3. Residue	90
	4. Supernatant	25

* Samples contained 14 μg chlorophylls and 250 μg proteins.
† Samples contained 28 μg chlorophylls and 450 μg proteins.

presumed "DNA" which was not solubilized by DNAase, from fraction 3, the total residue left after buffer action.

It is apparent that the isolated chloroplasts contain significant amounts of DNA. It also appears that under these experimental conditions the isolated chloroplasts contained little interfering substances, for most of the positively reacting substances in the DNA fluorescence reaction were also solubilized by DNAase.

It also appears that the DNA/protein ratio was not a constant and is probably dependent on the physiological state of the organelles. The ratio of DNA/protein obtained from experiment A was about 1/4,000, while the ratio from experiment B was 1/6,000.

To verify the localization of the DNA in the chloroplasts, the isolation procedure was modified. After the preliminary centrifugation to eliminate the broken cell walls, 0.4-ml samples of the green supernatant were layered over 4.4 ml of a continuous density gradient of buffered sucrose in each of three centrifuge tubes. The density gradient tubes were prepared by the procedure of Britten and Roberts,[14] the sucrose solution graded from 45 per cent sucrose in 0.01 *M* Tris buffer, pH 7.4, to 15 per cent sucrose in the same buffer. The tubes were centrifuged in the Spinco SW-39 rotor for 4 hr at 40,000 rpm. After centrifugation, a small hole was punched in the bottom of each tube, avoiding the green pellet, and equal aliquot fractions were collected into a series of 11 test tubes. The green pellets left in the centrifuge tubes were resuspended and pooled. (This is fraction 1, Fig. 2.) The parallel test tubes from the three series were also pooled. The pooled fractions were analyzed for chlorophylls and DNA by the procedures described above except that the DNAase treatment was omitted (Fig. 1). All fractions were examined by phase microscopy. The heavy green pellet (fraction 1) was found to be composed of clumps of broken and intact chloroplasts. The light chloroplast fractions (fractions 10, 11, 12, in Fig. 2) consisted of intact chloroplasts. A typical result of such an experiment is presented in Figure 2.

The direct correlation between the DNA distribution and the distribution of the chloroplasts is evident from this figure.

Discussion.—The use of enucleated cell fragments from which the plastids were isolated eliminates the possibility of contamination by nuclear DNA.

To demonstrate that the removal of the lower portions always results in the removal of the nucleus, the fate of a large number of cells so treated was followed.

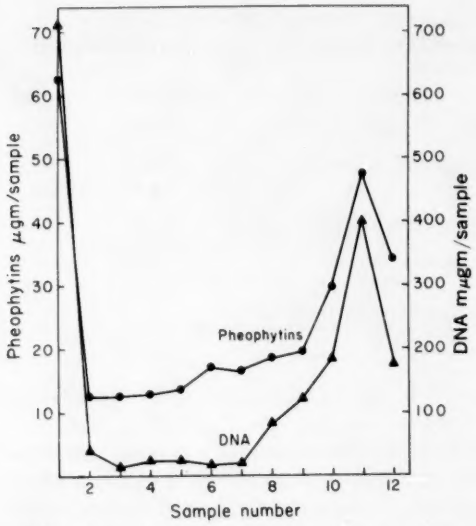

FIG. 2.—DNA and pheophytin distribution in fractionated cytoplasm of Acetabularia.

The basal (nucleated) portions continued to grow, whereas all the top, enucleated portions died after several weeks.

Use of bacteria-free cultures avoids the possibility of foreign DNA contaminating the preparations.

The correlation of the DNA distribution with the distribution of the chloroplasts in a density gradient substantiates the claim that the DNA is associated with the chloroplasts themselves.

The chloroplasts of Acetabularia are cylindrical bodies about 3 μ long and 1 μ in diameter, thus of an average volume, 3 μ^3. Assuming a composition of the chloroplasts such that the proteins comprise 20 per cent of the organelles, on a fresh-weight basis, the protein per plastid will amount to 6×10^{-13} gm. For a protein-to-DNA ratio of 6,000 to 1, the DNA content per plastid will be 1×10^{-16} gm. This corresponds to about 18×10^4 nucleotides. Assuming a triplet-type code and double-stranded DNA, this amount is sufficient for coding 3×10^4 amino acids or the synthesis of several hundred different proteins of an average molecular weight of 20,000.

The estimated value of 1×10^{-16} gm DNA per plastid is comparable to the amount of DNA in some virus particles. Vaccinia virus, an especially large virus, contains about 2×10^{-16} gm DNA per particle.[15] The DNA content of a single bacterial cell (*Aerobacter aerogenes*) is about 2×10^{-15} gm.[16] DNA values for the nuclei of a variety of higher organisms were found to vary from 0.11×10^{-12} gm for a sponge cell to 168×10^{-12} gm for an amphibian cell.[17]

The low concentration of DNA in the chloroplasts emphasizes the hazards of contamination of chloroplast preparations with nuclear DNA when gross cell homogenates are being fractionated. With our Acetabularia preparations it was possible to overcome this difficulty and demonstrate clearly that the chloroplasts contain small but significant quantities of DNA.

Summary.—Isolated chloroplasts from enucleated, bacteria-free Acetabularia cells were found to contain DNA in the approximate ratio of protein to DNA of 5,000 to 1. DNA was determined by the specific fluorometric procedure of Kissane and Robins[11] before and after hydrolysis with DNAase.

Centrifugation in a density gradient showed that the distribution of DNA paralleled the distribution of the chloroplasts, indicating that the DNA was either associated closely with the plastids or was indeed a part of them.

Dr. A. E. Mirsky suggested to us that the determination of DNA by the method of Kissane and Robins could be of value for a study of chloroplasts. We are grateful to Drs. S. Granick and A. E. Mirsky for suggestions and encouragement.

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*ON THE MECHANISM OF ACTION OF ALDOSTERONE ON SODIUM TRANSPORT: THE ROLE OF PROTEIN SYNTHESIS**

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Communicated by Julius H. Comroe, Jr., October 9, 1963

In an earlier study of the action of aldosterone on Na^+ transport across the isolated urinary bladder of *Bufo marinus*, we found that: (1) in steroid-free media, 12 hr of incubation was needed to sensitize the bladder to aldosterone, (2) in substrate-depleted bladders, hormonal activity depended on exogenous substrate, and (3) in substrate-enriched media, the response was characterized by a latent period of 60–90 min, followed by a linear increase in Na^+ transport over a period of 4.5–6.5 hr.[1]

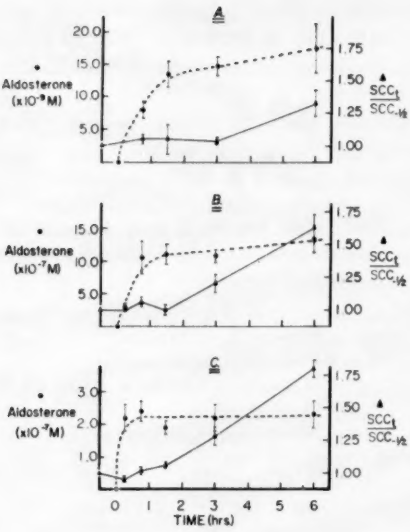

FIG. 1.—Uptake of H^3-aldosterone by the urinary bladder of toad. The ordinate on the left is the concentration of aldosterone (--●--) in bladder cells expressed in moles per liter of cell water. The ordinate on the right is the ratio of the short-circuit current (scc) recorded at time "t" after the addition of aldosterone to the medium, to the scc recorded $^1/_2$ hr prior to addition of aldosterone (—▲—). Time zero is the time of addition of aldosterone to the medium. Each point is an average of values from 6 to 8 hemibladders. The vertical lines represent ±1 standard error of the mean (s.e.m.). $A = 6 \times 10^{-9}$ M aldosterone in the serosal medium; $B = 7 \times 10^{-7}$ M aldosterone in the serosal medium; $C = 7 \times 10^{-7}$ M aldosterone in the mucosal medium.

Evidence will be presented to support the hypothesis that aldosterone is preferentially localized in the nucleus of the epithelial cells and acts by promoting DNA-dependent RNA synthesis that yields an increased rate of protein synthesis. The newly synthesized proteins appear to be enzymes involved in the coupling of metabolism to Na^+ transport.

Methods and Results.—Time-course of uptake of H^3-aldosterone: After double pithing of *Bufo marinus*, urinary bladders were removed and rinsed with frog-Ringer's solution (Na^+ = 114 mEq/l, K^+ = 3.5 mEq/l, Ca^{2+} = 5.4 mEq/l, Cl^- = 120.4 mEq/l, HCO_3^- = 2.5 mEq/l, Osmol. = 0.228, and pH in air = 8.4) containing 1.7×10^{-5} M mecholyl chloride. The hemibladders were mounted as diaphragms between glass chambers similar to those of Ussing and Zerahn.[2] Residual mecholyl chloride was removed by washing with 30 ml of frog-Ringer's solution for 15 min. All hemibladders were incubated overnight in 40 ml of frog-Ringer's solution (steroid-free) containing penicillin G (0.1 mg/ml). The following morning all solutions were replaced with frog-Ringer's solution fortified with glucose (5.5×10^{-3} M). Two hours later, 4 μC of 1,2 H^3- aldosterone[3] (specific activity = 50 mC/mg; repurified chromatographically by Dr. E. Biglieri) was added to either the serosal or mucosal solution of one hemibladder and diluent was added to the media of the other hemibladder. The final concentration of aldosterone was adjusted by adding cold d-aldosterone to the stock solution. Active Na^+ transport was measured by the short-circuit current (scc) method of Ussing and Zerahn.[2] The justification for equating Na^+ transport and scc in this system has been given previously.[1, 4]

Pairs of hemibladders were removed from the chambers at 0.25, 0.75, 1.50, 3, and 6 hr after the addition of aldosterone, and their water content was determined by drying for 24 hr at 95°C. The dried hemibladders were digested in 1.0 N KOH at 56°C, decolorized with H_2O_2, dissolved in 15 ml of scintillation so-

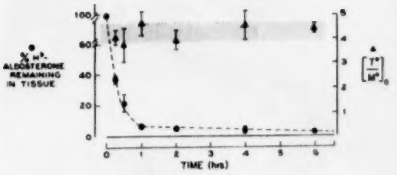

FIG. 2.—Elution of H^3-aldosterone from prelabeled hemibladders. The ordinate on the left is the per cent of H^3-aldosterone in the tissue (—●—) as a function of the time after incubation in steroid-free media. The ordinate on the right is the ratio of radioactivity in the tissue (T^*) to that of the medium (M^*) at the time of incubation in the steroid-free media (i.e., time zero). The stippled area covers 2 s.e.m. of (T^*/M^*) at time zero (i.e., gives the control value prior to incubation for all of the 44 hemibladders included in this study and shows that at the time of incubation in steroid-free media each group had about the same (T^*/M^*)). The length of the vertical bars is ±1 s.e.m.

lution, and assayed in a liquid scintillation spectrometer.[5] The intracellular concentration of aldosterone was calculated from the specific activity of the aldosterone, the radioactivity of the tissue, the water content of the tissue, and an assumed extracellular volume of 40%.[6]

At low concentrations of aldosterone in the serosal medium (i.e., 6×10^{-9} *M*), intracellular accumulation of aldosterone reached a steady-state value of 1.4×10^{-8} *M* in 90 min (Fig. 1*A*). In contrast, Na^+ transport was unaffected for 3 hr and then increased by 30% over the next 3 hr. At a concentration of aldosterone of 7×10^{-7} *M* in the serosal medium, intracellular accumulation achieved a steady-state value of 1.2×10^{-6} *M* in about 1 hr. The latent period for scc was 90 min, followed by a linear increase over the ensuing 4.5 hr (Fig. 1*B*). At a concentration of 7×10^{-7} *M* in the mucosal medium, intracellular accumulation of aldosterone achieved a steady-state value of 2.2×10^{-7} *M* in 15 min. The latent period for scc was between 60 and 90 min, followed by a linear increase in scc for 5 hr (Fig. 1*C*). These results suggest that the latent period is not a consequence of a slow rate of penetration of aldosterone and that the time-course of action is a result of a relatively slow set of intermediate reactions. The 12 hr of preincubation needed to sensitize the system to aldosterone may be dictated by the rate of decay of the intermediates.

The time-course of elution of H^3-aldosterone from prelabeled bladders: Paired hemibladders were immersed 3–14 hr in frog-Ringer's solution containing 1,2 H^3-aldosterone (0.5 μC/ml). The hemibladders were then rinsed for 30 sec, mounted in chambers, and incubated in steroid-free Ringer's solution 0.25–6 hr. The method of drying, digestion, and radioassay was described in the first section.

Ninety per cent of the aldosterone was eluted from the bladders in 1 hr, and 95%

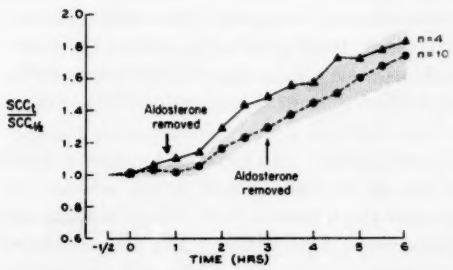

FIG. 3.—Effect of "premature" removal of aldosterone on the response of the scc. The ratio $scc_t/scc_{-1/2}$ is defined in the legend of Fig. 1. The group in which aldosterone was removed from the media 45 min after it was added is denoted by —▲—. The group in which aldosterone was removed 3 hr after its addition is denoted by —●—. The stippled area covers the mean ± 2 s.e.m. of the response of 26 hemibladders to continuous exposure to a constant concentration of aldosterone for 6 hr. *n* denotes the number of experiments in each group.

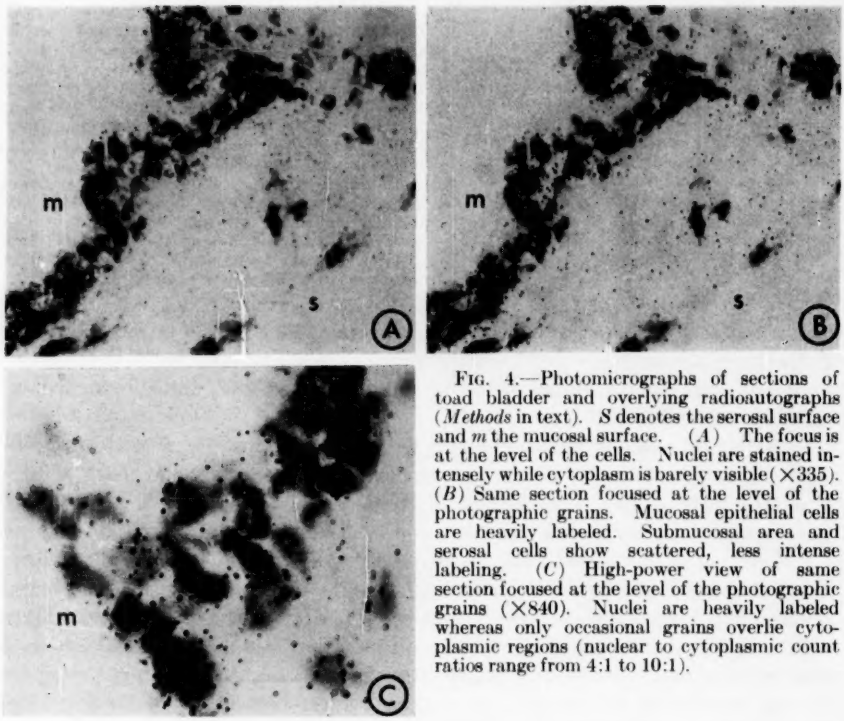

FIG. 4.—Photomicrographs of sections of toad bladder and overlying radioautographs (*Methods* in text). *S* denotes the serosal surface and *m* the mucosal surface. (*A*) The focus is at the level of the cells. Nuclei are stained intensely while cytoplasm is barely visible (×335). (*B*) Same section focused at the level of the photographic grains. Mucosal epithelial cells are heavily labeled. Submucosal area and serosal cells show scattered, less intense labeling. (*C*) High-power view of same section focused at the level of the photographic grains (×840). Nuclei are heavily labeled whereas only occasional grains overlie cytoplasmic regions (nuclear to cytoplasmic count ratios range from 4:1 to 10:1).

in 6 hr (Fig. 2). Therefore, the time required for sensitization to aldosterone is not a function of the total amount of aldosterone in the tissue. Steroidal action via intermediates can also be tested by removing aldosterone prior to the onset of the effect.

Effect of "premature" removal of aldosterone: In these experiments, aldosterone was added to the serosal medium (7×10^{-7} *M*) at time zero (i.e., 2 hr after the addition of glucose). In four experiments, the aldosterone-containing medium was exchanged for steroid-free frog-Ringer's solution at the end of 45 min and in 10 experiments, at the end of 3 hr. The results are given in Figure 3. The latent period and the rate of rise of the scc was the same when aldosterone was removed "prematurely" (i.e., either 45 min or 3 hr after it was added) as when aldosterone was present throughout. These results are consistent with the hypothesis that aldosterone acts via intermediates. Alternatively, these results may reflect the existence of a small, tightly bound pool of aldosterone at the site of active transport. To choose between these alternatives, we studied the site of intracellular localization of H^3-aldosterone by radioautography.

Localization of the site of binding of H^3-aldosterone: In these experiments, 2 μC of 1,2 H^3-aldosterone was added to the serosal solution 2 hr after the addition of glucose to the media. Ninety minutes later the bladders were removed from the chambers, blotted briefly, and plunged into liquid propane (−170°C). Sections 6 μ in thickness were cut on an International cryostat microtome and exposed to

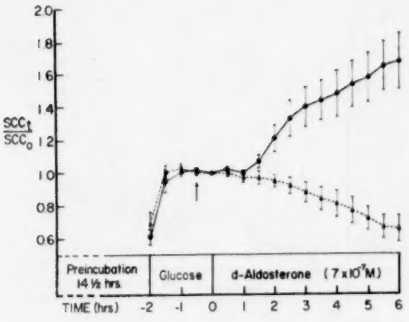

FIG. 5.—Effect of puromycin on action of aldosterone on Na^+ transport. scc_t denotes short-circuit current recorded at time "t" after addition of aldosterone; scc_o, short-circuit current recorded at time of addition of aldosterone (time zero). Arrow indicates time of addition of puromycin (80 μgm/ml) to media of one of each pair of hemibladders (--▲--). The control group (—●—) shows the usual response to aldosterone. Vertical bars represent ± 1 s.e.m.

formalin fumes. The sections were coated with Kodak liquid NTB 3 emulsion by the loop method to minimize aqueous extraction of the labeled steroid.[7, 8] The coated sections were exposed for ½–6 months at 4°C and developed with Kodak D-19. Some of the developed preparations were stained by the Bélanger method.[9]

Preferential localization was found in the mucosal epithelial cells (Fig. 4*A* and *B*). The serosal mesothelial cells were also labeled. The submucosal connective tissue showed sporadic, diffuse labeling; only background granules were found in the smooth muscle bundles and blood vessels. High magnification revealed selective localization over the nuclear and perinuclear areas of the mucosal epithelium (Fig. 4*B* and *C*). In contrast, only occasional loci of cytoplasmic labeling were noted.

In view of the physiological findings indicative of an indirect action of aldosterone, selective nuclear accumulation of the labeled steroid takes on considerable significance. Previous studies have shown that puromycin, an inhibitor of protein synthesis, and actinomycin, an inhibitor of nuclear synthesis of RNA, effectively block the cortisone-induced rise in liver tryptophan pyrrolase, tyrosine transaminase, and glycogen deposition.[10–12] As a test of the possibility that aldosterone also acts by enhancing protein synthesis via DNA-mediated RNA synthesis, we studied the effects of puromycin and actinomycin D on the action of aldosterone.

Effects of puromycin and actinomycin D on the action of aldosterone: Paired hemibladders were prepared as in the preceding experiments. In eight experiments, puromycin was added to the serosal and mucosal media (80 μgm/ml) of one hemibladder and an equal volume of the diluent to the media of the other 30 min before adding d-aldosterone (7×10^{-7} *M*) to the serosal solutions of both hemibladders. Six hours after adding aldosterone, vasopressin was added to the serosal solutions of both hemibladders (0.1 vasopressor units/ml). Pretreatment with puromycin abolished the effect of aldosterone on the scc (Fig. 5). The time-course of the scc in hemibladders exposed to puromycin plus aldosterone was the same as in the steroid-free media observed in an earlier study.[1]

In eight experiments, actinomycin D (20 μgm/ml) was added to the serosal and mucosal solutions of one hemibladder and diluent to the media of the other shortly after mounting the bladders. About 15 hr later, the solutions were exchanged for glucose-fortified solutions containing the same concentration of actinomycin D or diluent as in the preincubating solutions. Two hours after this exchange d-aldosterone was added to the serosal media of both hemibladders (7×10^{-7} *M*). Six hours after adding aldosterone, both hemibladders were challenged with vaso-

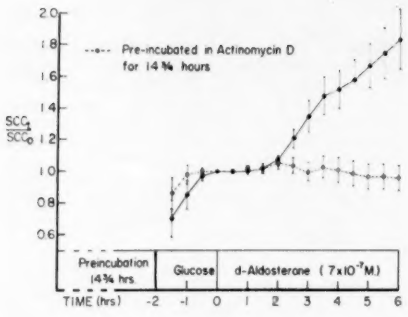

FIG. 6.—Effect of actinomycin the D on action of aldosterone. The ratio scc_t/scc_0 is defined in the legend of Fig. 5. The hemibladders pretreated with actinomycin D(—O—) for $14^3/_4$ hr show no obvious response to aldosterone. The controls (—●—) show the usual response.

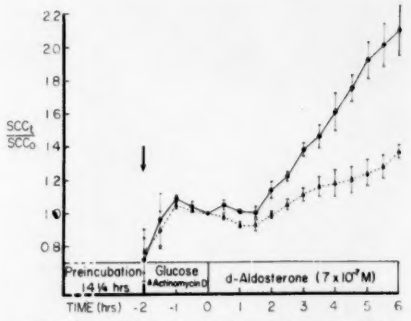

FIG. 7.—Effect of short exposure (2 hr) to actinomycin D on the action of aldosterone. The ratio scc_t/scc_0 is defined in the legend of Fig. 5. The arrow indicates the time of addition of actinomycin D or diluent. The hemibladders pretreated with actinomycin for 2 hr (--▲--) show a markedly inhibited response to aldosterone. The controls (—●—) show the usual response.

pressin (0.1 U/ml). The effect of aldosterone on the scc was reduced considerably in the hemibladder treated with actinomycin. The control hemibladder showed the usual response to aldosterone (Fig. 6). A comparison of these results with those obtained in steroid-free media indicates that actinomycin D inhibited the response to aldosterone by about 90 per cent.[1]

In three experiments, one of each pair of hemibladders was exposed to actinomycin D (20 μgm/ml) for only 2 hr prior to the addition of aldosterone. Pretreatment with actinomycin for 2 hr significantly reduced (by about 60%[1]) the effect of aldosterone on the scc (Fig. 7). The usual response to aldosterone was seen in the control hemibladder.

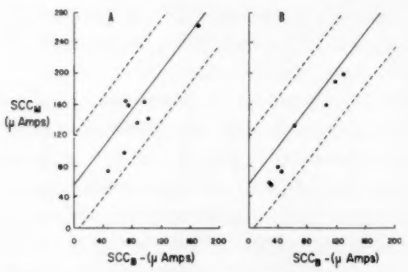

FIG. 8.—Action of vasopressin on Na^+ transport in presence of puromycin or actinomycin D. The maximum value of the scc (scc_M) after the addition of vasopressin (0.1 U/ml) to the serosal medium is plotted as a function of the baseline value (scc_B) just before the addition of the hormone. The regression line (——) is the mean response of 26 control hemibladders to the same concentration of vasopressin. The broken lines (– – –) represent 2 standard errors of estimate of scc_M. The experimental points in *A* indicate the response to vasopressin in the presence of puromycin (80 μgm/ml) and in *B* the response in the presence of actinomycin D (20 μgm/ml).

Three lines of evidence argue against the possibility that puromycin or actinomycin D produce nonspecific inhibition of the Na^+ transport system: (1) The rate of decay of the scc was no greater in the hemibladders exposed to puromycin or actinomycin D plus aldosterone than in a comparable group of hemibladders in steroid-free media.[1] (2) The response of the scc to glucose was about the same in actinomycin D-treated hemibladders as in the controls (Fig. 6). (3) The response of the scc to vasopressin was unaltered by puromycin or actinomycin D (Fig. 8).

These results provide direct support for the hypothesis that aldosterone acts by inducing *de novo* synthesis of

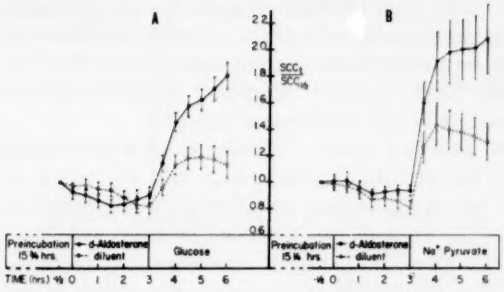

FIG. 9.—Effect of pretreatment with aldosterone on the response of Na^+ transport to substrate. Scc_t and $scc_{1/2}$ are defined in the legend of Fig. 1. The hemibladders pretreated with aldosterone (—●—) show a marked enhancement of response to both glucose at $5.5 \times 10^{-3} M$ (13 paired experiments) and pyruvate at $5.5 \times 10^{-3} M$ (9 paired experiments) compared with the hemibladders in steroid-free media (--○--).

protein. In L cells, Franklin and Baltimore[13] found partial inhibition of protein synthesis after 2 hr of exposure to actinomycin D and almost complete inhibition after 10 hr of exposure. Actinomycin has been shown to block DNA-dependent RNA synthesis by binding to DNA and specifically inhibiting the activity of RNA polymerase.[13–16] Puromycin inhibits synthesis of protein in intact cells and cell-free systems at the ribosomal level.[13, 17–20]

The effect of pretreatment with aldosterone on the response to substrates: If aldosterone acts by inducing *de novo* synthesis of enzymes involved in the coupling of metabolism to Na^+ transport, in substrate-depleted bladders a reversal of the sequence (i.e., pretreatment with aldosterone followed by the addition of substrate) should result in a curvilinear rise in the rate of Na^+ transport and should eliminate the latent period. In these experiments, 30 min after exchanging the media for substrate-free, frog-Ringer's solution, d-aldosterone ($7 \times 10^{-7} M$) was added to the serosal medium of one hemibladder and diluent to the serosal medium of the other. Three hours after the addition of aldosterone or diluent, either glucose or sodium pyruvate ($5.5 \times 10^{-3} M$) was added to the serosal and mucosal media of both hemibladders.

Pretreatment of substrate-depleted hemibladders with aldosterone had little effect on the scc after 3 hr (Fig. 9). The introduction of glucose or pyruvate produced a dramatic curvilinear rise in scc without a latent period. Thirty minutes after the addition of either substrate, the rise in the scc of the aldosterone-treated hemibladders was significantly greater than in the controls. The synergism between pyruvate and aldosterone suggests that aldosterone induces the formation of enzymes involved in the oxidation of pyruvate (Fig. 9*B*).

Discussion.—That aldosterone acts by inducing *de novo* synthesis of enzymes coupling metabolism to Na^+ transport is supported by the following results: (1) Aldosterone uptake reaches a steady level prior to the onset of its action. (2) Aldosterone is rapidly eluted into steroid-free media, whereas 12–14 hr are needed to sensitize the toad bladder to exogenous steroid. (3) "Premature" removal of aldosterone from the medium (i.e., 45 min) has no effect on the time-course of action on Na^+ transport. (4) Aldosterone is selectively localized in the nuclei of the receptor epithelial cells. (5) Actinomycin D or puromycin blocks the action of aldosterone but not the action of vasopressin on Na^+ transport. (6) Pretreatment of substrate-depleted bladders with aldosterone followed by the addition of substrate eliminates the latent period and yields a curvilinear rather than a linear rise in Na^+ transport.

The period of preincubation of 12–14 hr in steroid-free media needed for sensitization of the system to aldosterone presumably reflects the rate of turnover of the induced enzymes. The dependence on substrate presumably reflects the role of the induced enzymes in providing energy for Na^+ transport. The latent period presumably reflects the time required for *de novo* synthesis of enzymes.

In general, steroid action falls into two classes: regulation of physiological processes (e.g., Na^+ transport or carbohydrate metabolism), and regulation of cellular proliferation and maturation (e.g., vaginal cornification). Both classes of effects appear to be exerted at the level of DNA control of RNA synthesis. The findings of Feigelson *et al.*[10] and Greengard *et al.*[11, 12] indicate that cortisone acts to induce formation of hepatic tryptophan pyrrolase and tyrosine transaminase. Similarly, convincing evidence has been obtained recently to show that estrogen acts on the vagina and uterus and that testosterone acts on the testis by stimulating protein synthesis via an effect of nuclear synthesis of RNA.[21–24]

Summary.—The hypothesis is presented that aldosterone regulates active Na^+ transport by increasing *de novo* synthesis of enzymes involved in supplying energy for transport work. The evidence presented in support of this hypothesis included observations on: (1) the relationships between the uptake of H^3-aldosterone or its rate of removal and the time-course of action, (2) nuclear localization of H^3-aldosterone, (3) specific inhibition of the action of aldosterone by actinomycin D and puromycin, and (4) elimination of the latent period by pretreatment of substrate-depleted bladders with aldosterone.

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COMPLEMENTARITY BETWEEN LAMBDA (λ) PHAGE AND ESCHERICHIA COLI

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The nature of the λ prophage attachment to the *E. coli* genome is as yet unknown. Several models have been proposed which involve: (1) linkage of λ DNA to *E. coli* DNA by a protein bond;[1] (2) insertion of λ DNA into the host genome following the formation of a formally circular prophage structure which then undergoes a reciprocal crossover with the bacterial genome;[2] and (3) a nucleotide sequence homology between the λb_2^+ segment and a part of the host genome.[3] The last model was based on the fact that λb_2 deletion mutants cannot lysogenize *E. coli* K-12 although they can elicit the lytic response.

As a first step toward gaining some definite information concerning the nature of prophage attachment, the possible existence of homology between λ and *E. coli* was studied chemically. To date, the most sensitive chemical assay for polynucleotide complementarity relies upon the formation and detection of DNA-DNA[4, 5] or DNA–RNA hybrids.[6] The latter assay was chosen because of the accuracy and simplicity of the hybrid detection method.[7] In brief, our approach involved forming synthetic λ RNA of high specific radioactivity by the DNA-dependent RNA polymerase, and testing this RNA for its ability to combine specifically with *E. coli* DNA. A method for estimating the fraction of the λ genome that is homologous to *E. coli* DNA will be presented, as well as evidence that most, if not all, of the homology resides outside of the λb_2^+ region.

Materials and Methods.—Bacteria: *E. coli* strains W3110 and W3110 (λ) (derivatives of strain K-12) were obtained from Dr. J. Weigle, and strain B/5 from Dr. R. Edgar. *E. coli* were grown in M9 medium[8] fortified with 1.5% casamino acids (Difco). *Shigella dysenteriae* 15, obtained from Dr. M. Lieb, were grown in nutrient broth containing glucose and bactopeptone.

Phage: T4 phage were obtained from Dr. R. Edgar. $\lambda b_2^+ b_5 c$ were isolated by CsCl density gradient centrifugation[9] following a cross between wild-type λ and $\lambda b_2 b_5 c$ (obtained from Dr. J. Weigle). The phage were treated with 2.5 γ/ml DNAase for 1 hr at 37° after concentrating them by centrifugation. When used for DNA preparations, the phage were suspended in CsCl (Harshaw, optical grade) at a density of 1.50 gm/cc and centrifuged at 33,000 rpm for at least 18 hr. The phage band was isolated by collecting drops through the bottom of the tubes.[10] The phage preparation was then dialyzed against 0.15 *M* NaCl, 0.015 *M* Na_3 citrate (1 × SSC).

DNA preparations: Phage DNA was prepared by the phenol method of Grossman *et al.*[11] For use as primer for RNA synthesis, it was dialyzed against 0.01 *M* tris, pH 7.9, 0.06 *M* KCl. Otherwise, the DNA was dialyzed against SSC/100.

Bacterial DNA was isolated from log-phase cultures at $\sim 5 \times 10^9$ cells/ml. Cells were lysed by the freeze-thaw-lysozyme-Duponol method,[12] and DNA purified by repeated extractions with $CHCl_3$- isoamyl alcohol (24/1, v/v[13]). Generally the DNA was not separated from RNA as it

was found unnecessary. Removal of over 99% of the RNA from one *E. coli* DNA preparation had no effect on the amount of synthetic λ RNA capable of hybridizing with a given amount of DNA.

RNA preparations: The DNA-dependent RNA polymerase was isolated according to the procedure of Chamberlin and Berg.[14] The reaction mixture for preparing the synthetic λ and T4 RNA[14] contained H^3-ATP at a specific activity of 1.40 c/mM (Schwarz BioResearch, Inc.). Native DNA was used to prime the reaction, which was allowed to proceed for 1 hr at 37°C. The RNA was extracted two times with ice-cold phenol saturated with 0.01 *M* tris, pH 7.4, ether extracted to remove the phenol, then dialyzed for 18–24 hr against 1 × SSC at 5°C. The enzyme was apparently free of *E. coli* DNA. In the absence of primer DNA, no RNA was synthesized that could hybridize with *E. coli* DNA.

Unlabeled coli RNA, 30 sec pulse-labeled coli RNA, and λ-infected coli RNA were isolated from strain W3110. The bacteria were grown with aeration to 4×10^8 cells/ml in K medium containing 0.5% glycerol as carbon source instead of glucose so as to facilitate the attachment of λ.[15] The culture was concentrated 10-fold by centrifugation and resuspension in fresh 37° medium, then divided into three flasks. With no aeration, one culture was infected with $\lambda b_2{}^+ b_5 c$ at a multiplicity of 7 phage/cell. After 5 min, each culture was diluted 10-fold with fresh medium at 37°, and aeration was resumed (= zero time). After 8 min, 1.5 μc/ml H^3-uracil (New England Nuclear Corp., 3.54 c/mM) was added to the second culture. At $t = 8.5$ min, incorporation was stopped by the addition of 1/100 volume 1 *M* NaN_3, and each culture was dumped onto 0.4 vol. frozen 0.01 *M* tris, pH 7.4. The cells were spun down, resuspended in 1/100 vol. 0.01 *M* tris, pH 7.4, 0.005 *M* $MgCl_2$, 0.01 *M* NaN_3, and frozen.

Cells were lysed as described previously,[12] and RNA was extracted with 37° phenol saturated with 0.05 *M* Na acetate, pH 5.2, 0.005 *M* $MgCl_2$, 0.06 *M* KCl. The RNA was dialyzed against 1 × SSC for 12 hr at 5°C.

Formation and detection of DNA-RNA hybrids: DNA samples in SSC/100 were denatured by boiling for 15 min, then rapidly cooling to 0°C.[4, 5] The DNA-RNA hybrid reaction was carried out in 2 × SSC for 4 hr at 61°C. The solution was then transferred to 37° and incubated for 10 min with 6 γ/ml pancreatic RNAase (Worthington Biochemical Corp.; previously heated at 98°C for 10 min to eliminate DNAase). The amount of RNA complexed to DNA was determined by the filtration method of Nygaard and Hall.[7] The RNAase treatment completely eliminated RNA nonspecifically complexed by high DNA concentrations. The background level of RNA trapped by the filter in the absence of any DNA or of homologous DNA was equal to ~0.5% of the total RNA added. This level could not be lowered by filtering the RNA prior to its use in the hybrid reaction. At DNA concentrations below 1 γ/ml, ~30% of the λ RNA complexed to λ DNA was removed in the first min of RNAase digestion, and none in the next 9 min. Similar observations with coli DNA-RNA hybrids were also reported by Yankofsky and Spiegelman.[16] The exact nature of the RNAase-sensitive "hybrid" is unknown.

Measurements of "effective" DNA concentration: The amount of RNA that is *detectably* complexed with DNA is a function of both the DNA concentration in the reaction and the fraction of DNA capable of being trapped by the filter membranes. The latter could conceivably vary for different DNA preparations. Each DNA preparation used was therefore tested for its "trapping efficiency." A sample was-heat denatured, its salt concentration raised to 2 × SSC, and filtered after measuring the O.D. at 260 mμ with a Zeiss spectrophotometer. The membrane was washed as for hybrid measurements, and the UV absorption of the eluate determined. The difference between the input and the eluted absorption represents the fraction of material bound. This procedure was checked by the diphenylamine assay for DNA,[17] and was found to be an accurate measure of the DNA absorbed by the filter membrane. For one *E. coli* nucleic acid preparation, 11% of the total O.D. units was capable of being trapped on the filter after denaturation. The diphenylamine assay indicated that 10% of the total UV-absorbing material was DNA. It thus appears that essentially all of the *E. coli* DNA was capable of being trapped. The same was true for λ DNA. DNA concentrations were determined by multiplying the $O.D._{260}$ of the preparation by the fraction absorbable by the filter membrane, and converting this value to μg/ml by the factor 50 (extinction coefficient = 20 cm^2/mg).

Results.—(1) *Demonstration of homology between λ and E. coli:* DNA-RNA hybrids were formed using a range of DNA concentrations and a constant amount

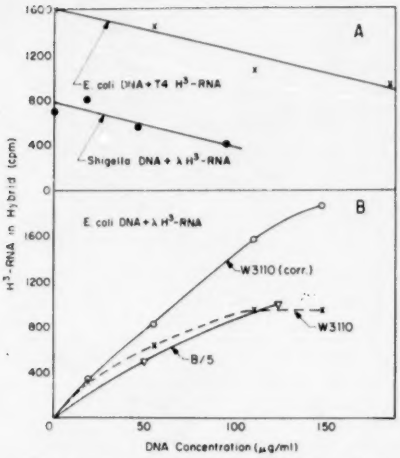

FIG. 1.—Specificity of the hybrid reaction. Hybrids were formed and assayed as described in *Methods*. In 1*B*, the λH³-RNA background was 487 cpm for the reactions with *E. coli* W3110 DNA and 371 cpm with *E. coli* B/5 DNA. The background was proportional to RNA input, and, except for self-absorption, independent of DNA concentration. The correction for self-absorption (1*B*) was based on the negative slope of the appropriate DNA (1*A*). Taking the value at zero DNA as 100% (no self-absorption), the per cent self-absorption could be determined at any DNA concentration, and the observed hybrid cpm thus corrected.

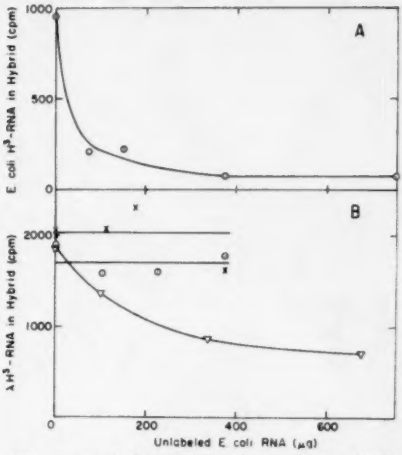

FIG. 2.—(*A*) Competition between *E. coli* H³-RNA and *E. coli* RNA for *E. coli* DNA sites (theoretically 100% competition). Reaction mixtures contained 58 μg coli H³-RNA, 27 μg coli DNA, and the designated amount of cold coli RNA in 0.44 ml. Hybrids were assayed as described in *Methods*. (*B*) Failure of *E. coli* RNA to compete with $\lambda b_2^+ b_5 c$ H³-RNA (x–x) and $\lambda b_2 b_5 c$ H³-RNA (o–o) for *E. coli* DNA sites. Each reaction mixture contained 0.22 and 0.26 μg H³-RNA, respectively, 52 μg coli DNA, and the indicated amount of cold coli RNA in a final volume of 0.33 ml. The ability of synthetic $\lambda b_2^+ b_5 c$ H³-RNA to compete for λ DNA sites with $\lambda b_2^+ b_5 c$ RNA synthesized *in vivo* is also shown (Δ–Δ). Each reaction contained 0.22μg $\lambda b_2^+ b_5 c$ H³-RNA, 2μg $\lambda b_2^+ b_5 c$ DNA, and the indicated amount of $\lambda b_2^+ b_5 c$-infected coli RNA in 0.44 ml. The observed hybrid cpm were 10-fold greater than indicated.

of H³-RNA for each type of DNA tested. This method provides a more sensitive assay for the existence of homology than simply choosing one arbitrary set of DNA and RNA concentrations.

As shown in Figure 1*B*, enzymatically synthesized λ H³-RNA was able to combine about as well with RNA from either *E. coli* W3110 or *E. coli* B/5. The specificity of hybridization of the synthetic λ H³-RNA is seen (Fig. 1*A*) by its inability to combine with Shigella DNA. The latter possesses a base composition similar to that of *E. coli* DNA[18] and also shows considerable homology with *E. coli* DNA.[19] Shigella is not susceptible, however, to infection by λ, and cannot be lysed or lysogenized when λ DNA is injected by mating with *E. coli*(λ).[20] The specificity of the *E. coli* DNA was demonstrated by its failure to combine with synthetic T4 H³-RNA.

The negative slope in the cases where no homology exists can be explained by the increase in self-absorption of the weak H³ β emission with increasing amounts of DNA on the filter membrane. The same slope resulted whether the DNA was present during the hybrid reaction or added just prior to filtration. A correction for self-absorption is illustrated in Figure 1*B*.

It must be stressed that the declaration of no homology between T4 and *E. coli* and between λ and Shigella is based on an operational definition. It means that no RNAase-insensitive RNA remained bound to DNA after a hybrid reaction at 61°C. The minimal chain length of a DNA-RNA complex that would be stable to these conditions is unknown.

Further evidence for base sequence complementarity between *E. coli* and λ was obtained from a study of the melting profile of the coli DNA-λRNA hybrid.[21]

(2) *Significance of the λ-E. coli homology:* Before concluding definitely that there is homology between λ and *E. coli*, we must rule out the possibility that the λ DNA preparation is contaminated by *E. coli* DNA. If this were indeed the case, the DNA used to prime the synthesis of λ H^3-RNA would also cause the formation of *E. coli* H^3-RNA, which could then account for the observed homology with coli DNA. The likelihood of this possibility was reduced by using a λ_c mutant incapable of lysogenizing *E. coli*, and by the extensive purification of the phage. The absence of *E. coli* H^3-RNA in the reaction mixture was demonstrated by the following RNA competition experiments.

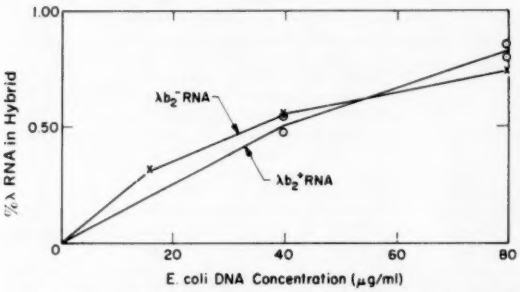

FIG. 3.—Homology outside of the λb_2^+ segment of the genome. The reaction mixtures contained the indicated concentration of coli DNA and either 138,000 cpm of $\lambda b_2^+ b_5 c$ H^3-RNA or 147,000 cpm of $\lambda b_2 b_5 c$ H^3-RNA in 0.22 ml. The total input cpm were determined by acid precipitation on membrane filters. The amount of RNA hybridized was assayed as described in *Methods*. The percentage of hybridized RNA was determined after subtracting the appropriate RNA background.

Increasing amounts of unlabeled *E. coli* RNA synthesized *in vivo* did not compete significantly with $\lambda b_2^+ b_5 c$ H^3-RNA or $\lambda b_2 b_5 c$ H^3-RNA for sites in *E. coli* DNA (Fig. 2*B*). The ability of this preparation of coli RNA to compete for sites in coli DNA with coli H^3-RNA synthesized *in vivo* is shown in Figure 2*A*.

One might then question whether cellular RNA is able to compete with RNA synthesized *in vitro* by the DNA-dependent RNA polymerase. To answer this, enzymatically synthesized λ H^3-RNA was hybridized with λ DNA in the presence of various amounts of RNA isolated from λ-infected *E. coli*. As seen in Figure 2*B*, over 60 per cent of the synthetic λ H^3-RNA could be displaced from λ DNA sites by competition with λ RNA synthesized *in vivo*.

It may therefore be concluded that the λ DNA was not contaminated by a random sampling of *E. coli* DNA. These experiments do not eliminate the possibility of the presence of one or a few coli genes in λ phages, since little competition would occur between the resulting synthetic coli RNA and the heterogeneous coli RNA added. This problem, however, approaches one of semantic significance, for such a specific coli gene "contamination" might well be considered an integral part of the λ phage.

(3) *Genetic site(s) of homology:* The b_2^+ region of the λ phage genome has been suspected of being homologous to *E. coli*,[3] primarily because it is necessary for

lysogenizing *E. coli* K-12 although unnecessary for the formation of mature phage particles.[22] λb_2 deletion mutants are lower in density than wild-type λ[22] and have been shown to lack about 18 per cent of the DNA.[3] The hybrid test was therefore carried out with RNA synthesized using $\lambda b_2 b_5 c$ DNA as primer in order to determine whether any homology exists between *E. coli* DNA and λ RNA lacking the b_2^+ region. The results are shown in Figure 3.

Approximately the same percentage of $\lambda b_2^+ b_5 c$ RNA and $\lambda b_2 b_5 c$ RNA is capable of combining with *E. coli* DNA. Thus, there is a region outside of the b_2^+ section of the λ genome which is homologous to *E. coli* DNA. These results do not exclude the possibility, however, that there is also some coli homology in the b_2^+ region. Since approximately the same percentage of each type of RNA (λb_2^+ and λb_2) is homologous to coli DNA, and the λb_2 genome is only 82 per cent of the λb_2^+ genome, as much as one fifth of the total coli-homologous λ DNA could reside in the b_2^+ region. This calculation is based on the assumption that the b_2^+ region is transcribed into RNA to the same extent as other regions of the λ genome.

(4) *What fraction of the λ genome is homologous to E. coli DNA?* A fixed amount of synthetic $\lambda b_2^+ b_5 c$ H^3-RNA was incubated with varying concentrations of DNA from λ, *E. coli*(λ), and *E. coli*. The amount of RNA hybridized was plotted as a function of DNA concentration (see Fig. 4). Using the λ DNA as a standard for comparison, since it is completely homologous to the λ RNA, it is possible to estimate the concentration of λ-homologous DNA present in a reaction mixture. Knowing the input DNA concentration, the λ-homologous fraction of that DNA can be determined.

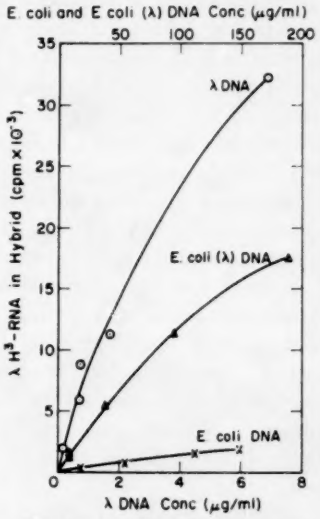

FIG. 4.—Fraction of the λ genome homologous to *E. coli* DNA. Each reaction mixture contained 156,000 cpm of $\lambda b_2^+ b c$ H^3-RNA and the designated concentration of $\lambda b_2^+ b_5 c$ DNA, *E. coli*(λ) DNA, or *E. coli* DNA in 0.44 ml final volume. Hybrids were assayed as described in *Methods*. Self-absorption corrections were made as described in Fig. 1.

The average ratio of *E. coli*(λ) to λ DNA at various points along the curves was 64. Thus, 1.6 per cent (1/64) of the *E. coli*(λ) genome is comprised of λ DNA and possible λ-homologous coli DNA. Taking the molecular weight of an *E. coli* genome as 2.5×10^9,[23] and assuming one prophage per genome, the amount of λ-homologous DNA in the host genome is $\sim 4 \times 10^7$ daltons. The molecular weight of vegetative λ DNA is 3.1×10^7.[24]

A fixed incubation time (4 hr) was used for each hybrid reaction, so it was necessary to establish the fact that the presence of *E. coli* DNA did not affect the rate of formation of λ DNA-RNA hybrids. This was found to be the case using 100 times as much *E. coli* DNA as λ DNA in the reaction mixture.

By comparing the amount of *E. coli* DNA equivalent to *E. coli*(λ) DNA (Fig. 4), it was possible to estimate the fraction of the λ genome homologous to *E. coli* DNA. For two preparations of *E. coli* DNA, coli DNA/coli(λ) DNA, ratios of 1/15 and

1/11 were obtained. Thus, ~13 times more *E. coli* DNA than *E. coli*(λ) DNA is required in order to hybridize the same amount of λ RNA. This result indicates that 1/13, or 7.5 per cent of the λ genome, is present in the nonlysogenic *E. coli* genome. The region of DNA common to each is therefore about 2.3×10^6 daltons ($7.5\% \times 3.1 \times 10^7$), or about 6700 nucleotides.

The accuracy of this estimate is based primarily on the assumption that the coli-homologous region of λ DNA is transcribed into RNA by the RNA polymerase as readily as other regions of the genome. At present, there is no reason for doubting this, while there is some indication that it is true since the entire DNA molecule appears to be transcribed *in vitro*.[14, 25]

Discussion.—The existence of a considerable amount of homology (~6700 nucleotides) between λ phage and its bacterial host suggests that some role important to both organisms must be prescribed by the common region. Two properties of the λ-*E. coli* system might well be expected to require homology. One is the ability of λ to transduce selectively the *gal* genes of *E. coli*. The transducing λ particle has been shown to be missing a large portion of its own genome (~1/3–1/4),[26] which has been replaced by the *E. coli gal* region, possibly by a genetic recombination involving homology. A second property which seems likely to require homology is the ability of λ to establish a stably lysogenic cell with the prophage possessing a specific site on the host genome.[27]

If there were homology between λ DNA and the *gal* region of *E. coli*, the same would most likely exist between λ and Shigella, which shows considerable chemical[28] and genetic[20] homology with *E. coli*. However, no homology was observed between λ and Shigella. Studies on the formation of transducing λ phages led Campbell to the conclusion that little homology exists between λ and the *gal* region.[29] We are thus drawn to the tentative conclusion that complementarity between λ and *E. coli* DNA is required for the establishment of a stably lysogenic cell.

The λb_2^+ region is required in order for λ to be able to lysogenize *E. coli* K-12.[3] It has thus been suspected of being homologous to the prophage site on the *E. coli* genome. The demonstration that most, if not all, of the homology lies outside of this segment is not surprising, however, in view of the work of Arber and Lataste-Dorolle.[30] Several strains of *E. coli* B, transduced for λ sensitivity, were shown by these authors to be capable of being stably lysogenized by the λb_2 deletion mutant.

It has been shown recently that the replication of the *E. coli* genome proceeds unidirectionally,[23, 31, 32] and that the λ prophage is replicated at a time corresponding to its position.[32] On the basis of known molecular weights,[23, 24] λ DNA is 1.24 per cent of the weight of an *E. coli* genome. The demonstration that 1.6 per cent of the *E. coli*(λ) genome is λ DNA (*Results*, section 4) indicates that the λ genome replicates before the majority of *E. coli* genes in this strain (W3110). Moreover, it can be concluded that both strands of λ DNA are present in the prophage state.

The calculation that ~8 per cent of the λ genome is homologous to *E. coli* DNA (*Results*, section 4) is not subject to the uncertainty of the time of prophage replication. This result was based on the comparison of *E. coli* DNA and *E. coli*(λ) DNA with respect to their ability to combine with λ RNA. Since the ratio of λ prophages to λ-homologous *E. coli* DNA regions is presumably unity in the same *E. coli*

strain, no correction factor is necessary. As mentioned previously, the 8 per cent value does rely on the ability of the RNA polymerase to transcribe the coli-homologous region of the λ genome to the same extent as other regions.

In contrast to our results, Cowie and McCarthy have very recently reported that 33 per cent of the λ genome is homologous to *E. coli* DNA.[33] Their method entailed measuring the amount of λ DNA which could saturate *E. coli* DNA trapped in agar. The following criticisms of their estimation of homology must be considered:

(1) *E. coli* phosphodiesterase was not used after the hybrid reaction to remove bound, nonhybridized DNA. As seen from the studies of Schildkraut *et al.*,[34] enzyme treatment is necessary to obtain distinct hybrid bands in the CsCl gradient.

(2) That λ DNA was nonspecifically bound is also likely since only 45 per cent of the λ DNA that eluted from the complex with *E. coli* DNA was capable of hybridizing with *E. coli* DNA a second time. Since the DNA-agar column was stated to be 80 per cent efficient in binding λ DNA, presumably a maximum of 56 per cent (45/80) of the original λ DNA bound was actually homologous to *E. coli* DNA.

(3) Saturation of the agar-bound *E. coli* DNA with λ DNA indicated that 0.2 per cent of the *E. coli* genome was homologous to λ DNA. Taking the *E. coli* genome as 2.5×10^9 daltons,[23] 5×10^6 daltons or 16 per cent of the λ genome appears to be homologous to *E. coli* DNA. This value should then be corrected, since only 56 per cent of this binding can be considered as true homology, leaving 9 per cent of the λ genome homologous to *E. coli* DNA.

The experiment shown in Figure 2*B* indicates that over 60 per cent of the λ RNA synthesized *in vitro* could be displaced from λ DNA sites by means of competition with λ RNA synthesized in *E. coli* 0–$8^1/_2$ min after infection. If both strands of λ DNA were uniformly transcribed into RNA *in vitro*,[14, 25] this result, if accurate, would indicate that more than one strand was transcribed into λ-specific RNA *in vivo*. This topic is currently being investigated.

Summary.—From DNA-RNA hybrid studies, it was possible to conclude that about 8 per cent of the λ genome is complementary to the DNA of *E. coli* strains W3110 and B/5. At least 80 per cent of this homology lies outside of the λb_2^+ segment of the genome. There is no homology between λ and *Shigella dysenteriae*, as defined experimentally. Various lines of evidence support the view that the observed homology is required for the formation of the lysogenic state rather than for transduction.

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ERGODIC THEORY, GROUP THEORY, AND DIFFERENTIAL GEOMETRY

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1. *Introduction.*—In this note we shall show how two programs announced earlier may be extended and partially unified by means of the concept of an "ergodic groupoid." The first of these programs[1] began with a generalization of the Laplace transform to locally compact commutative groups and involved a notion of analyticity for functions defined on the direct product of a connected locally compact commutative group with the vector space of all of its one-parameter subgroups. Some ten years later an extension of this first program was described[2] in which the dependence on group theory was removed, certain connections with ergodic theory were brought out, and differential notions other than analyticity were included. The second program grew out of our work on the problem of relating the unitary representations of a noncommutative separable locally compact group to those of a normal subgroup. The "ergodic case" of this problem suggests that every ergodic action of a separable locally compact group G be regarded as defining a "virtual

subgroup" of G which defines the action just as a closed subgroup H of G defines the transitive action of G on G/H. As described briefly on pages 652–654 of the notes to our 1961 colloquium lectures,[3] one can develop a theory of virtual subgroups which is parallel in many ways to the theory of locally compact groups.

The notion of ergodic groupoid (defined below) allows us to define a virtual group as a "similarity class" of ergodic groupoids and develop a theory of generalized groups which does not require them to occur as (virtual) subgroups of conventional groups. An ergodic groupoid for which every element is uniquely determined by its left and right units is said to be principal. Principal ergodic groupoids may be thought of as "ergodic equivalence relations." Refining and generalizing the notion of C^∞ ergodic lacing (defined in ref. 2), we are led to a notion which differs from that of C^∞ manifold in having "extra global structure." This extra global structure is defined by an ergodic equivalence relation, and the theory of virtual groups is applicable to it.

2. *Ergodic Groupoids and Equivalence Relations.*—Let S be a set. Let $\mathcal{E}$ be a subset of $S \times S$ which defines an equivalence relation in S. Let us introduce a multiplication in $\mathcal{E}$ by setting $(s_1,s_2)(s_3,s_4) = (s_1,s_4)$ whenever $s_2 = s_3$ and declaring the product to be undefined when $s_2 \neq s_3$. With this (not everywhere defined) multiplication, $\mathcal{E}$ satisfies all of the axioms for a Brandt groupoid[4] except the final one stating that any two units are the left and right units of a single element. It will be convenient to change Brandt's terminology (as has been done by others) and use the term "groupoid" to denote the more general object obtained by omitting the final axiom. (A groupoid may also be defined as an abstract category in which every map has an inverse.) The units of $\mathcal{E}$ are just the pairs s,s and so correspond one to-one to the elements of S. Moreover, $s_1,s_2 \in \mathcal{E}$ if and only if s_1,s_1 and s_2,s_2 are the left and right units, respectively, of the same element of $\mathcal{E}$. Thus, the pair $S,\mathcal{E}$ is determined by $\mathcal{E}$ and its groupoid structure. $\mathcal{E}$ is a *principal* groupoid in the sense that for a given ordered pair of units there is at most one groupoid element having these as left and right units, respectively. Clearly every principal groupoid is obtainable in this way from an equivalence relation. Now let $\mathfrak{F}$ be any groupoid which is also a (an analytic) Borel space.[5] We shall say that $\mathfrak{F}$ is a (an analytic) Borel groupoid if the two structures are so related that (*i*) the domain of definition $\mathfrak{D}$ of the product is a Borel subset of $\mathfrak{F} \times \mathfrak{F}$, (*ii*) $z_1, z_2 \to z_1z_2$ is a Borel function from $\mathfrak{D}$ to $\mathfrak{F}$, (*iii*) $z \to z^{-1}$ is a Borel function from $\mathfrak{F}$ to $\mathfrak{F}$. In any groupoid zz^{-1} is the left unit of z, and $z^{-1}z$ is the right unit of z. Thus, if $\pi(z) = zz^{-1}$, then π is a Borel function mapping $\mathfrak{F}$ onto the set $S_{\mathfrak{F}}$ of all units, and $S_{\mathfrak{F}}$ is a Borel subset of $\mathfrak{F}$. Let $\psi(z) = \pi(z),\pi(z^{-1})$. Then ψ is a Borel map of $\mathfrak{F}$ onto a subset of $S_{\mathfrak{F}} \times S_{\mathfrak{F}}$ which defines an equivalence relation in $S_{\mathfrak{F}}$. The mapping ψ is one-to-one if and only if $\mathfrak{F}$ is a principal groupoid and in any case is a homomorphism of $\mathfrak{F}$ onto the principal groupoid defined by the indicated equivalence relation. If G is a separable locally compact group and G acts on S so as to convert it into an analytic Borel G space, then $S \times G$ becomes an analytic Borel groupoid if $(s_1,x_1)(s_2,x_2)$ is defined when and only when $s_1x_1 = s_2$ and then is equal to (s_1,x_1x_2). This groupoid is principal if and only if $sx = s$ implies $x = e$ whenever $s \in S$ (e is the identity of G.)

Let μ be a finite measure[6] in the analytic Borel groupoid $\mathfrak{F}$. Let $\tilde{\mu}(E) = \mu(\pi^{-1}(E))$ for all Borel subsets E of $S_{\mathfrak{F}}$. As is well known,[7] there exists an assignment (unique almost everywhere with respect to $\tilde{\mu}$) of a measure μ_s to each $\pi^{-1}(s)$ such

that for every Borel set $F \subset \mathfrak{F}$ we have $\mu(F) = \int_{S_\mathfrak{F}} \mu_s(F \cap \pi^{-1}(s)) d\tilde{\mu}(s)$. Changing μ to another measure with the same null sets does not alter the null sets of $\tilde{\mu}$ or those of the μ_s. Thus, every measure class[8] C in $\mathfrak{F}$ defines a unique measure class $\tilde{C}$ in $S_\mathfrak{F}$ and (almost everywhere) a unique measure class C_s in $\pi^{-1}(s)$. Notice now that for each z in $\mathfrak{F}$, the mapping $x \to zx$ is a one-to-one Borel function from $\pi^{-1}(z^{-1}z)$ to $\pi^{-1}(zz^{-1})$. If there exists a null set N in $S_\mathfrak{F}$ such that for all z in $\mathfrak{F}$ with $zz^{-1} \epsilon N$ and $z^{-1}z \epsilon N$ this map carries the measure class $C_{z^{-1}z}$ onto the measure class $C_{zz^{-1}}$, we shall say that C is left invariant. If C is left invariant and invariant under $z \to z^{-1}$, we shall say that it is invariant. An invariant C will be said to be *ergodic* if no real valued Borel function f on S which is not $\tilde{C}$ almost everywhere constant can have the property that $f(z^{-1}z) = f(zz^{-1})$ for C almost all z in $\mathfrak{F}$. If C is invariant and ergodic, then the pair $\mathfrak{F}, C$ will be called an *ergodic groupoid*. If the ergodic groupoid $\mathfrak{F},C$ is principal (so that $\mathfrak{F}$ is defined by an equivalence relation in $S_\mathfrak{F}$), we may obtain a measure class C_s' in the equivalence class of s by applying the mapping $z \to \pi(z^{-1})$ to the measure class C_s in $\pi^{-1}(s)$. Because C is invariant it follows that $C_{s_1}' = C_{s_2}'$ whenever s_1 and s_2 are equivalent. Thus, an invariant measure class C in $\mathfrak{F}$ defines a measure class $\tilde{C}$ in $S_\mathfrak{F}$ and a measure class in each equivalence class; conversely, C and the C_s' determine C. When we wish to emphasize these facts about principal ergodic groupoids, we shall call them *ergodic equivalence relations.*

THEOREM 1. *Let G be a separable locally compact group and let S be a standard Borel G space. Make $S \times G$ into a Borel groupoid as indicated above. Let C_1 be a measure class in S and let C_2 be the measure class of Haar measure in G. Then $C_1 \times C_2$ is invariant with respect to the groupoid structure of $S \times G$ if and only if C_1 is invariant in S under the G action. If $C_1 \times C_2$ is invariant, then it is ergodic if and only if C_1 is ergodic in S under the G action.*

THEOREM 2. *Let $\mathfrak{F}$, C be an ergodic groupoid and let S_0 be a Borel subset of $S_\mathfrak{F}$ of positive $\tilde{C}$ measure. Let $\mathfrak{F} \restriction S_0$ denote the set of all $z \epsilon \mathfrak{F}$ with $\pi(z) \epsilon S_0$ and $\pi(z^{-1}) \epsilon S_0$. Then C restricted to $\mathfrak{F} \restriction S_0$ converts it into an ergodic groupoid which is principal whenever $\mathfrak{F}$ is principal.*

Theorems 1 and 2 assure us of the existence of many examples of ergodic groupoids and equivalence relations. If the S_0 of Theorem 2 differs from S by a set of $\tilde{C}$ measure zero, we shall call $\mathfrak{F} \restriction S_0$ an *inessential contraction* of $\mathfrak{F},C$.

3. *Virtual Groups.*—Let $\mathfrak{F}_1,C_1$ and $\mathfrak{F}_2,C_2$ be ergodic groupoids and let ϕ be a Borel function from $\mathfrak{F}_1$ to $\mathfrak{F}_2$. We shall say that ϕ is a *strict homomorphism* if (*i*) $\phi(z_1)\phi(z_2)$ is defined and equal to $\phi(z_1z_2)$ whenever z_1z_2 is defined, and (*ii*) if $S_{\mathfrak{F}_2}$ does not have a single equivalence class whose complement is of C_2 measure zero, then $\tilde{\phi}^{-1}(E)$ is a C_1 null set whenever E is a Borel subset of $S_{\mathfrak{F}_2}$ which is a C_2 null set. $\tilde{\phi}$ denotes the restriction of ϕ to $S_{\mathfrak{F}1}$. We shall say that ϕ is a *homomorphism* if its restriction to some inessential contraction is a strict homomorphism. Let ϕ be a (strict) homomorphism from $\mathfrak{F}_1,C_1$ to $\mathfrak{F}_2,C_2$ and let ψ be a (strict) homomorphism from $\mathfrak{F}_2,C_2$ to $\mathfrak{F}_3,C_3$. Then the composite mapping $\psi \circ \phi$ is easily seen to be a (strict) homomorphism of $\mathfrak{F}_1,C_1$ to $\mathfrak{F}_3,C_3$. We shall say that the strict homomorphisms ϕ_1 and ϕ_2 of $\mathfrak{F}_1,C_1$ into $\mathfrak{F}_2,C_2$ are strictly similar[9] and write $\varphi_1 \approx \varphi_2$ if there exists a Borel map θ of $S_{\mathfrak{F}1}$ into $\mathfrak{F}_2$ such that for all s in S the right and left units of $\theta(s)$ are $\phi_1(s)$ and $\phi_2(s)$, respectively, and $\theta(zz^{-1})\phi_1(z) = \phi_2(z)\theta(z^{-1}z)$ for all z in $\mathfrak{F}_1$. We shall say that two homomorphisms ϕ_1 and ϕ_2 are similar and write $\phi_1 \sim \phi_2$ if they have strictly similar restrictions to a common inessential contraction of $\mathfrak{F}_1,C_1$. It

is not hard to show that similarity and strict similarity are equivalence relations and that $\psi_1 \circ \phi_1$ is (strictly) similar to $\psi_2 \circ \phi_2$ if ϕ_1 and ψ_1 are (strictly) similar to ϕ_2 and ψ_2, respectively. Let ϕ_1 be a homomorphism of $\mathfrak{F}_1,C_1$ into $\mathfrak{F}_2,C_2$ and let ϕ_2 be a homomorphism of $\mathfrak{F}_2,C_2$ into $\mathfrak{F}_1,C_1$. If $\phi_2 \circ \phi_1$ and $\phi_1 \circ \phi_2$ are both similar to the relevant identities, we shall call the pair ϕ_1,ϕ_2 a *similarity* of $\mathfrak{F}_1,C_1$ with $\mathfrak{F}_2,C_2$ and say that the two ergodic groupoids are similar. By a *virtual group* we shall mean a similarity class of ergodic groupoids. Similarities between ergodic groupoids "commute" with similarities between homomorphisms. Consequently, we may think of a similarity class of homomorphisms as being a homomorphism between the corresponding virtual groups. The following two theorems are actually special cases of a general theorem about homomorphisms of ergodic groupoids into groups. The general theorem will be formulated and proved in one of the detailed papers which will follow this announcement.

THEOREM 3. *Let H_1 and H_2 be closed subgroups of the separable locally compact groups G_1 and G_2. Let S_j denote the coset space G_j/H_j and convert $S_1 \times G_1$ and $S_2 \times G_2$ into ergodic groupoids as above. Then these ergodic groupoids are similar if and only if H_1 and H_2 are isomorphic as topological groups. Moreover, the similarity classes of homomorphisms of $S_1 \times G_1$ into $S_2 \times G_2$ correspond one-to-one in a natural way to the conjugacy classes of continuous homomorphisms of H_1 into H_2.*

THEOREM 4. *Let G be a separable locally compact group and let C_1 and C_2 be ergodic invariant measure classes in the analytic Borel G spaces S_1 and S_2. Let $\mathfrak{F}_j$ denote the groupoid associated with $S_j \times G$ so that $\mathfrak{F}_j,C_j$ is an ergodic groupoid for $j = 1,2$. Let ϕ_j denote the homomorphism $s,x \to x$ of $\mathfrak{F}_j,C_j$ into G. Suppose that there exists a similarity ψ_1,ψ_2 of $\mathfrak{F}_1,C_1$ with $\mathfrak{F}_2,C_2$ such that ϕ_1 is similar to $\phi_2 \circ \psi_1$. Then there exist invariant Borel null sets N_1 and N_2 in S_1 and S_2, respectively, and a one-to-one Borel mapping f of S_1-N_1 on S_2-N_2 such that $f(sx) = f(s)x$ for all $s,x \in S_1$-$N_1 \times G$.*

It follows from Theorem 4 that each ergodic action of G is determined to within an obvious equivalence by a certain virtual group and a homomorphism of this virtual group into G; thus, so to speak, by a "virtual" subgroup of G. Theorems 3 and 4 lend support to the view that virtual groups constitute a natural generalization of locally compact groups and may be expected to share many of their properties. The author plans to develop the theory of virtual groups in some detail in subsequent publications.

4. *Measures in Ergodic Equivalence Relations.*—Let $\mathcal{E}$ be a Borel subset of $S \times S$ defining an equivalence relation in the analytic Borel space S and let C be a measure class in $\mathcal{E}$ converting it into a principal ergodic groupoid. Let μ be a σ finite member of C and let ν be any σ-finite member of $\bar{C}$. Applying the decomposition theorem referred to above to a finite member of C and working in an obvious manner with Radon Nikodym derivatives, one finds measures μ_s in the $\pi^{-1}(s)$ such that $\mu = \int \mu_s d\nu(s)$. The μ_s are determined uniquely almost everywhere by μ and ν; changing ν merely changes each μ_s by a multiplicative constant. We shall say that the pair μ,ν is *invariant* if $\mu_{s_1} = \mu_{s_2}$ for almost all pairs s_1,s_2 in $\mathcal{E}$, and μ is carried into itself by the map $s_1,s_2 \to s_2,s_1$.

THEOREM 5. *If μ,ν is an invariant pair, then μ and ν determine one another up to a multiplicative constant. If μ,ν is an invariant pair and σ is any positive Borel function, then μ',ν' is an invariant pair where $\nu'(E) = \int_E \sigma(s) d\nu(s)$ and $\mu'(F) = \int_F \sigma(s_1)\ \sigma(s_2) d\mu(s_1 s_2)$.*

COROLLARY. *Let $s \to \mu_s^\circ$ be the assignment of measures to the equivalence classes of S defined by an invariant pair μ,ν. Then μ is determined by this assignment up to a multiplicative constant. Choosing this constant once and for all one has a "natural" one-to-one correspondence between the possible assignments $s \to \mu_s^\circ$ and the members ν of $\bar{C}$.*

5. *A Generalization of C^∞ Manifolds.*—Let $S,\mathcal{E}$ be as in the preceding section and let each equivalence class in S under $\mathcal{E}$ be given the structure of a C^∞ manifold. Let $\mathfrak{R}$ be the ring of all real valued Borel functions on S whose restriction to each equivalence class is a C^∞ function. Assume the given assignment to be such that $\mathfrak{R}$ separates points so that the assignment is actually determined by $\mathfrak{R}$. Let $C_s^{\mathfrak{R}}$ denote the measure class in the equivalence class of s defined by its C^∞ manifold structure, that is, the class whose restriction to each sufficiently small neighborhood is that of the measures defined by the nonzero n forms (where n is the dimension of the manifold). Let C be a measure class in $\mathcal{E}$. We shall say that the quadruple $S,\mathcal{E},C,\mathfrak{R}$ is a *C^∞ ergodic equivalence relation* if $S,\mathcal{E},C$ is an ergodic equivalence relation which admits an invariant pair and for $\bar{C}$ almost all s in S we have $C_s = C_s^{\mathfrak{R}}$.

One can construct a large class of examples as follows. Let $S,\mathcal{E},C$ be obtained from the action of a group G as indicated in Theorem 2. Let G be a Lie group and let $\bar{C}$ contain a measure which is invariant under the group action. Then each equivalence class is a G orbit and inherits a C^∞ structure from that of G. It is not obvious that the ring $\mathfrak{R}$ always separates points but it is easy to see that it does in numerous special cases, e.g., whenever S is a C^∞ manifold on which G acts smoothly.

Let $S,\mathcal{E},C,\mathfrak{R}$ be a C^∞ ergodic equivalence relation. Then each $s \in S$ is contained in a unique C^∞ manifold M_s. Let V_s denote the tangent space to M_s at s. We shall call V_s the *tangent space to S at s*. We may now define vector and tensor fields on S just as in ordinary differential geometry. The contravariant vector field L taking s into L_s will be said to be a *Borel vector field* if for every $f \in \mathfrak{R}$ the function $L(f)$ taking s into $L_s(f)$ is a Borel function on S. If $L(f)$ is a member of $\mathfrak{R}$, we shall say that L is a *C^∞ vector field*. We define Borelness and being C^∞ for other kinds of vector and tensor fields in an analogous fashion. Many notions of ordinary differential geometry extend in an obvious fashion to C^∞ ergodic equivalence relations. For example, if f is a member of $\mathfrak{R}$, then df is the C^∞ covariant tensor field (one form) such that $(df)_s(v) = f_v(s)$ where $v \in V_s$ and f_v is the action of v on f.

It is important to notice that a C^∞ ergodic equivalence relation is rather far from being the product of a measure space and a C^∞ manifold. This is partly because of the implications of ergodicity. For instance, let f be a member of $\mathfrak{R}$ such that $df = 0$. Then f must be constant on each equivalence class and hence, by ergodicity, must be constant almost everywhere on S. To illustrate the assertion of the introduction about "extra global structure," let our C^∞ ergodic equivalence relation be such that each component C^∞ manifold is diffeomorphic to E^n. Let W be a C^∞-one form such that $dW = 0$. Then on each equivalence class there exists a C^∞ function f with $df =$ restriction of W to the class. The f for each class is unique up to an additive constant. To find a member F of $\mathfrak{R}$ such that $dF = W$ we must choose these constants so that the resulting function on S is a Borel function on S. There are many examples showing that this is not always possible. Thus, the measure theoretic structure of $S,\mathcal{E},C$ can have the same kind of an effect on the global differential

geometry of $S,\mathcal{E},C,\mathcal{R}$ as a nonvanishing first Betti number has on that of a C^∞ manifold.

In the integration of differential forms we have another respect in which C^∞ ergodic equivalence relations have global properties transcending those of the component C^∞ manifolds. Let ω be a Borel n form on S where n is the common dimension of the equivalence classes. Let it be positive with respect to an orientation. Then ω defines a unique member ν_s of each C_s, and ν_s depends only upon the equivalence class of s. Since $S,\mathcal{E},C$ admits an invariant pair, the assignment $s \to \nu_s$ is associated with an essentially unique member of $\bar{C}$. Hence, modulo the choice of a single arbitrary constant, we obtain a natural one-to-one correspondence between the members of the measure class $\bar{C}$ on S and the positive Borel n forms on S. Hence, it makes sense to speak of the integral of an n form over S or any Borel subset of S. More generally we can define C^∞ maps of m dimensional C^∞ ergodic equivalence relations into n dimensional ones and integrate m forms over such maps. It seems likely that one can formulate and prove an analogue of Stokes' theorem.

Given a Riemannian metric in a C^∞ ergodic equivalence relation, one has a natural associated positive n form and hence (up to a multiplicative constant) a natural measure μ in S. The formal Laplace Beltrami operator defined by the metric carries with it a corresponding notion of harmonic function and defines a symmetric operator in $\mathcal{L}^2(S,\mu)$. Interesting questions arise concerning the relationship between the spectral properties of the operator, the existence of harmonic functions on S, and the nature of the associated ergodic equivalence relation. In a similar manner practically every branch of analysis which is concerned with relating local differential properties to global ones suggests a family of questions about C^∞ ergodic equivalence relations. We hope to investigate some of these questions in later publications.

6. *Applications to Ergodic Theory.*—An ergodic flow has associated with it an ergodic equivalence relation and hence a virtual group. In many (if not in all) cases it will also be associated with a unique one-dimensional C^∞ ergodic equivalence relation. Group theory and differential geometry thus suggest a vast array of questions to ask about flows. One can hope that one will discover useful invariants of flows in this fashion and thus make progress in the difficult problem of classifying ergodic flows.

7. *Remarks.*—(*a*) In measure theoretic questions it is often useful to eliminate null sets by formulating everything in terms of the Boolean algebra obtained by factoring out the ideal of null sets from the Boolean algebra of all Borel subsets of a given space. On the other hand, in thus banishing points, one loses a conceptual and technical aid of some importance. Our strategy at the moment is to postpone a serious attempt to formulate everything in terms of Boolean algebras until the theory has been further developed and is better understood in its present form. Nevertheless, it is useful to keep the Boolean algebra point of view in mind, especially when one is doubtful about the "right way" to define a concept involving exceptional null sets.

(*b*) One can of course adapt the definitions of sections 2 and 3 to the groupoids defined by G spaces and equivalence relation spaces S where S is a topological space rather than a measure space. We have not yet explored the consequences of taking this point of view in topology in any detail. However, we have come upon one

application which seems to be worth mentioning. Let $\mathfrak{U}$ be an open covering of a topological space X and let each member of the covering be such as to admit only trivial fiber bundles. Let X° denote the set of all pairs $\mathcal{O},x$ where $\mathcal{O} \in \mathfrak{U}, x \in \mathcal{O}$ and topologize X° so that for each fixed $\mathcal{O}$ the map $\mathcal{O},x \to x$ is a homeomorphism of its domain with $\mathcal{O}$ and the set of all $\mathcal{O},x$ is open in X°. Let us introduce an equivalence relation in X° by setting $\mathcal{O}_1,x_1$ equivalent to $\mathcal{O}_2,x_2$ whenever $x_1 = x_2$. Then X is homeomorphic to the space of all equivalence classes. Let $\mathcal{E}$ denote the set of ordered pairs defining this equivalence relation and regard $\mathcal{E}$ as a topological groupoid. Then the similarity classes of continuous homomorphisms of $\mathcal{E}$ into a topological group H correspond one-to-one in a natural way to the equivalence classes of principal bundles over X with group H. This fact is new at most in formulation. I am indebted to R. S. Palais for pointing out that it is simply a reformulation of the theorem connecting coordinate bundles and fiber bundles in section 2 of Steenrod's book.[10] On the other hand, it seems to be a useful way of looking at the connection in question and to be a suggestive way of thinking about fiber bundles and their properties. For example, if one decides to study the linear representation theory of the "virtual group" associated with the groupoid $\mathcal{E}$ and to construct the ring of all characters, one is led in a straightforward way to define the Grothendieck ring $K(X)$ of the space X. On the other hand, one must not press the group analogy too far in the case of similarity classes of topological groupoids. The ergodicity condition in section 3 plays a very important role, and similarity classes of groupoids are much less "grouplike" when it is missing.

(c) In his work on the foundations of differential geometry, C. Ehresmann has introduced a very general and abstract notion of local structure.[11] In defining and developing this notion he has been led to deal extensively with groupoids and to define topological groupoids, topological categories, and even groupoids and categories with additional structure defined by an abstract category. Though this work is different from ours in both aim and spirit (and moreover is not concerned with measure theory), there are points of contact. In particular, the device of turning a product $S \times G$ into a groupoid occurs in Ehresmann's work in the more general context in which G is a category "acting" on S and $S \times G$ is turned into another category. We are indebted to S. Sternberg for calling our attention to Ehresmann's work after reading a preliminary draft of this note. We are similarly indebted to R. J. Blattner for calling our attention to related work of Y. H. Clifton and J. W. Smith.[12] These authors are concerned with the following question. Let X be a manifold and let there be given a foliation of X. Let $\tilde{X}$ be the set of all leaves. Then the natural quotient topology in $\tilde{X}$ may be so degenerate that the only open sets are the empty set and the whole space. Can one find a substitute for $\tilde{X}$ that plays the role played by $\tilde{X}$ in the more tractable case in which the foliation is a fibering? Clifton and Smith propose a substitute which has a significant cohomology theory and reduces to $\tilde{X}$ when the foliation is a fibering. Now a C_∞ ergodic equivalence relation is a sort of foliation in which one has replaced the topological structure in the containing space by a measure theoretic one. The associated virtual group has space-like properties—in particular a cohomology theory. To the extent that this virtual group plays the role of the "topological object" of Clifton and Smith, there is a parallel between part of section 5 of this paper and the work of these authors. On the other hand, our virtual group is certainly different

from their topological object—even in their context—since it does not depend on any topological structure in the underlying space. Moreover, it is possible that there are further differences. A closer study of the relationship would seem to be desirable. Apart from the parallel just described, the program announced in section 5 seems to have little in common with that apparently contemplated by Clifton and Smith.

* Part of the work announced in this note was done while the author was a fellow of the John Simon Guggenheim Foundation.

[1] Mackey, G. W., "The Laplace transform for locally compact abelian groups," these PROCEEDINGS, **34,** 156–162 (1948).

[2] Mackey, G. W., "The Laplace transform on groups and generalized analytic functions," Seminars on Analytic Functions, Air Force Office of Scientific Research, vol. 2 (1958), pp. 259–272.

[3] Published as "Infinite dimensional group representations," by G. W. Mackey, *Bull. Am. Math. Soc.*, **69,** 628–686 (1963).

[4] See Jacobson, N., *Theory of Rings* (New York: American Mathematical Society, 1943), pp. 132–133.

[5] See Mackey, G. W., "Borel structure in groups and their duals," *Trans. Amer. Math. Soc.*, **85,** 134–165 (1957), for definitions of this and related notions.

[6] In this note a measure is a countably additive set function taking values in the closed interval $0 \leq x \leq \infty$.

[7] See Theorem 11 of "Induced representations of locally compact groups, I," by G. W. Mackey, *Annals of Math.*, **55,** 101–139 (1952), for a formal statement with references to proofs.

[8] A measure class is the set of all σ finite measures having the same null sets as one of them.

[9] We have observed that a groupoid is a category with inverses. From this point of view a homomorphism is a functor, and (as pointed out to us by S. Eilenberg) strict similarity of homomorphisms is (apart from measure theoretic requirements) just natural equivalence of the corresponding functors.

[10] Steenrod, N. E., *The Topology of Fibre Bundles* (Princeton University Press, 1951).

[11] Ehresmann, C., "Gattungen von lokalen Strukturen," *Jber. Deutsch. Math. Verein,* **60,** 49–77 (1957), section 1.

[12] Clifton, Y. H., and J. W. Smith, "The category of topological objects," these PROCEEDINGS, **47,** 190–195 (1961); and "Topological objects and sheaves," *Trans. Amer. Math. Soc.*, 436–452 (1962).

CENTENNIAL CELEBRATION OF THE NATIONAL ACADEMY OF SCIENCES

FOURTH SCIENTIFIC SESSION

October 23, 1963

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INTRODUCTION

BY GEORGE B. KISTIAKOWSKY

HARVARD UNIVERSITY

Billions of years ago living matter on Earth profoundly, though unwittingly, altered its environment and thus made possible evolution of life into higher forms. As Dr. Wald so eloquently explained in the First Scientific Session, billions of generations of millions of species participated then in the conversion of the atmosphere from a reducing into an oxidizing medium.

We are living in an age when, for the second time, living matter can change its environment on a global scale. This time it is a single specie, *Homo sapiens*, in a period of time measured in tens rather than billions of generations, which is capable of altering the Earth's environment. Weather modification, global management of fresh water and of vegetation, utilization of mineral and energy resources, and manipulation of the continents' topography are powers which man possesses or will soon possess because of his knowledge of nature.

The urge to understand has been a salient characteristic of man since the beginnings of recorded history. Because of his unique ability to transmit experience and accumulate knowledge, which was stressed by Dr. Mayr in our Third Scientific Session, man has come into a position of dominance in the living world. The scientist's role in this process earned him a public position next to the philosopher,

artist, and poet, until after the time of the Renaissance when a new tool was perfected by the use of which he could project a new, more exclusive public image. This tool was the experimental method which led to the flourishing of natural science in the Western World.

Our Academy's birth occurred at a time when systematic use of accumulated scientific knowledge for practical purposes was first being made, which soon caused the flourishing of synthetic chemicals and metallurgical industries. Only recently did societies as a whole, through governments, recognize science as a powerful stimulus for political, economic, and cultural change and progress; and thus only recently did governments begin consciously to promote science. The result has been its continuing exponential growth, the appearance of many new scientific disciplines, and also the emergence of many difficult problems. President Kennedy spoke of some of the problems in his address at our Centennial Convocation. Some others will be considered by the distinguished speakers this afternoon.

The extraordinarily rapid growth of science (let us not forget that over 90 per cent of all natural scientists who ever lived are still alive) and the proliferation of new areas of research have led, from sheer volume as well as other factors, to difficulties in communication. There is the problem of transmitting information among the sciences, to preserve their coherence, if not unity; an individual scientist knows more and more about less and less. A far more difficult problem is communication between scientists and those who apply the results of scientific research to political, technological, and cultural purposes. This is the topic about which we shall hear from Dr. Oppenheimer.

Dr. Wiesner will speak of our Government's effort to expand science in the expectation of rich practical returns for our society and to enhance its cultural contributions.

Dr. Fisk will tell us of the problems which arise when knowledge obtained in basic research is being translated into practical uses by organized modern industrial research teams. These are the uses that constitute the progress of our technological civilization.

And finally Dr. Rabi will speak of science in the framework of the culture of a free society as a force of far deeper significance than mere satisfaction of material needs.

COMMUNICATION AND COMPREHENSION OF SCIENTIFIC KNOWLEDGE

BY J. ROBERT OPPENHEIMER

INSTITUTE FOR ADVANCED STUDY

The theme that has been assigned to me seems in some ways a little odd. That is only in part because this talk comes after three days and fifteen lectures in which, as actors and auditors, we have lived with many beautiful examples of good communication, and even very largely good comprehension—good understanding—of scientific knowledge. If I have any doubts, it may be that here and there, in those

reports which dealt with subjects close to me, the communication and the understanding have gone a little bit beyond the knowledge.

In an important sense, the sciences have solved the problem of communicating within and with one another more completely than has any human enterprise. I may retell an old story. Thirty-five years ago, Dirac and I were in Göttingen. He was making the quantum theory of radiation, and I was a student. He learned that I sometimes wrote a poem, and he took me to task, saying, "In physics we try to say things that no one knew before in a way that everyone can understand, whereas in poetry. . . ."

It is an old and consistent tradition with us to be concerned with the words we use, and with their purification, and thus with the concepts in terms of which we describe nature. It was true of Newton, of Lavoisier, of Cauchy, of Mendel, and of course, in our day, of Einstein, and of Bohr. As for Newton, we will understand this better when we have, after almost three centuries, the critical edition of the *Principia;* at least we will know that in the renowned *Hypotheses non fingo* it is not the first word but the last that bears the meaning.

When we tell about our work, we explain what we have done and we tell what we have seen, whether we are describing a radioastronomical object, or a new property of fiber bundles, or the behavior of men attempting to solve problems. We are prepared to believe that the explicit content of science has its roots in these accounts of action, often factual, often foreshortened and synoptic, because cast in terms which the scientific traditions have established long ago.

Among us there is surely a great and appropriate variation in how we describe this foundation for the objectivity of our knowledge, and for the lack of ambiguity in the terms we use to tell of it; and of course there is an even wider latitude, insofar as we may bring ourselves to speak of them, in what we think of the reasons for the success of science, in what attributes of the world of nature in which we find ourselves underlie the manifestations of order which are our business: why we can work on the same table and with the same test tube when we cannot have the same melancholy or the same resolution; why so much of the order of the natural world finds its expression in number and the more abstract mathematical structure.

We probably all, with varying enthusiasm, would say *yes* to Charles Peirce as to *how to make our ideas clear.* We would make a good case that we do indeed know the structure of some ribonucleic acids, or some properties of the longer-lived particles of physics, only leaving room for the fact that in new things as well as in old, there are points we may not have looked at, and that wonders may be hidden in the crevasses.

This foundation for knowledge precludes much that is an essential part of man's life. One cannot be a very effective scientist if he is a practicing solipsist. We cannot expect to describe a common world of introspection by telling people what we have done and what we have seen; though probably we can, and increasingly we will, describe elements of behavior which may have some correspondence to the inner world. Among these things of which we cannot talk without some ambiguity, and in which the objective structure of the sciences will play what is often a very minor part, but sometimes an essential one, are many questions which are not private, which are common questions, and public ones: the arts, the good life, the good society. There is to my view no reason why we should come to these with a

greater consensus or a greater sense of valid relevant experience than any other profession. They need reason, and they need a preoccupation with consistency; but only insofar as the scientist's life has analogies with the artist's—and in important ways it does—only insofar as the scientist's life is in some way a good life, and his society a good society, have we any professional credentials to enter these discussions, and not primarily because of the objectivity of our communication and our knowledge. But if I doubt whether we have a special qualification for these matters, I doubt even more that our professional practices should disqualify us, or that we should lose interest and heart in preoccupations which have ennobled and purified men throughout history, and for which the world has great need today. Your lives attest this.

This account of a constant concern within the scientific enterprise to purify and refine our language is, of course, a sort of parody of what we are all about. We do not really do this except in moments of crisis, or in order to make way for something very new and deep. We come to our new problems full of old ideas and old words, not only the inevitable words of daily life, but those which experience has shown fruitful over the years. This is an inevitable approach to the new; and when it is not too new, it gets by. But the comprehension, the understanding of scientific knowledge is a very different thing from being the recipient of a communication. I think there is an element of action inseparable from understanding: to question, to try, to apply, to adapt, to ask new questions, to see if one understands, and to test what one has been told: action in the laboratory or the observatory, or on paper, or, at the very least, in the motions of the spirit. We need, at times, to talk about the sources and the springs of this motion, without which communication would provide the fuel pipes, the electrical wiring, the transmission of a car, but not the combustion which gives it power and life.

We do not talk of this very well: imagination, play, curiosity, invention, action, these are all involved. They are indeed only rarely all combined, and supplemented by skepticism and criticism, in any one man in any one moment; one of the charms of the scientific enterprise is how deficient we can be in many of these qualities and still play some meaningful part in it.

We know that we love the old words, the old imagery, and the old analogies, and that we keep them for more and more unfamiliar and more and more unrecognizable things. Think of "wave," "information," "relativity." We know that one can explore and study the springs of the movement of science, that it is a fit if very difficult subject of study. Today at least we are not able to talk about it very well, not at all as we can of molecules or galaxies, or even of the effective definition of the words that we use. Yet we may be sure that without a living engagement there is no understanding and there is no life of science, and we know that we cannot command this, or perhaps even learn it, except by apprenticeship, by following what others have done, and by listening to the mischievous voices of adventure and play and exploration and doubt with which we greet a new experience or a new communication. This has very much to do with what we can in practice and honestly mean by the unity of science. I think, for instance, of contemporary mathematics, whose absence from this program does not at all reflect a lack of vitality, of discovery, and of beauty in the current scene. Up to our time, it has been the experience of our enterprise that there have been a good number of men

who combined creation and wide knowledge of the mathematics of their day with a lively interest in those elements of the natural sciences in which this mathematical order might be embodied. This conversation, as a lively mutual understanding, is rather thin today. It is not rare to find a physical scientist who will hear some beautiful new result—in algebra, for instance, or topology—with pleasure, with amazement, and with admiration; but it is not likely that he will be deeply engaged, and try to see if he can make it wider, how it affects other things he may have known, or thought to know. I know that it is also true that many mathematicians will accept with a certain interest that there are in nature two neutrinos which have different properties, or that astronomers believe that they may be witnessing evidence of very massive gravitational implosion in other galaxies. To me it seems good that we still do tell each other these pieces of news; but I would hope that the century-long tradition of a felt sense of reciprocal relevance between mathematics and natural science would soon again find itself embodied in many of us, or, far more plausibly, in our successors.

Thus between us, as specialists in our professions, there is a partly accidental quality to the effectiveness of our converse with one another, and thus to the effective unity of our view of the world, even as scientists. There are two reasons for deploring this. One is that past experience suggests so strongly that among the sciences there are elements of relevance and mutual enlightenment which make such converse an essential part of deep and rapid progress; the other is that we regret for ourselves what we do not really know, and we regret for others what we cannot really tell them. This is, of course, a reflection, within the internal society of the scientific enterprise, of a situation that characterizes our relations with human societies as a whole, with the society within which we are embedded, and that leaves us with problems, some very grave, and by no means all clearly soluble, having to do just with the communication and comprehension—understanding—of scientific knowledge.

These problems rest, of course, on human weakness and limitation; but more specifically they rest on at least three features of the scientific enterprise which it has in common with the world in which its whole action takes place: size and saturation, growth and change, and specialization. I will not speak to size, having no true wisdom as to whether there is a natural and appropriate limit to how vast the scientific enterprise can and should be, beyond which it suffers too deeply from suffocation and fragmentation. I do not think that I know an answer, but I rather hope that those who follow me today may have some wisdom; and I know that they have some views on how large our world should be, perhaps because that is a still harder question.

One thing we do know: growth and change imply size, and growth and change are very deep in the nature of the scientific enterprise. Without them we would not recognize the rooms in which we were living, or what our days were all about. As for specialization, it is what sharpens our tools and our words, and is the instrument of penetrating deeper and farther into the world of nature.

I think that we must live with these, and that we can live with them. Some of us will know one thing, some fewer will know many things, and the unity of our knowledge, its freedom from contradiction, and its important and often very deep

common relevance, will not preclude but will be enriched by the great and blessed diversity of man.

These limits on the communication and comprehension of scientific knowledge which we find among ourselves, with which we have been living and will continue to live, have their analogies in the related but vaster limitations that we have in our external relations with those who are not yet, or not ever, involved in the scientific enterprise. The first of these is with the young, those who may be entering the life of science, and perhaps also, perhaps even more importantly, those who may not. I cannot speak with even a decent record of experience of authority of the problems of education and schooling, for I have known them only at the late level that is essentially apprenticeship, where a young man or woman has become engaged in some part of science, and the problem is to help him enlarge his interest and his power and his knowledge of what others have done. I have the impression, which I hope may be true enough to be shared by most of you, that in the graduate schools, and in their increasing postdoctoral studies, we have in the natural and mathematical sciences rather happy arrangements for this period of apprenticeship, happy in comparison with the situation in other branches of study—historical or philosophical, for instance—rather happy in comparison with our sister institutions abroad, and very happy indeed in comparison with our own country some fifty years ago. Apart from this, my life gives me no qualification except to express an appreciation to our many colleagues who have been studying and practicing the teaching of the sciences in the schools and the colleges, so that first sight shall not repel, and the institutions not resist the natural curiosity and love and joy of the experience, but open it, so that as many as can will have an opportunity to discover some trait of nature, to see with welcome some sure sign of order in nature, with their own hands and their own heads.

I know that our colleagues understand the universal value, in all teaching, of quickly correcting error. I know that they are concerned to free the teaching of science of a slovenly and lazy dependence on history, in which discoveries were often made in obscure, contingent, and not deeply relevant struggles, whose interest as history is not helpful to the young student, and usually obscure to his teacher. I know that they hope, as often as may be, to open perspectives on the larger connections in nature and in the sciences which describe it, and rarely, when it can be done with historical scruple, on some chapters in the history of man's knowledge.

We mostly take it for granted, though it is not quite obvious, that we would like to have this opening of the world of science, this induction into it, effective not only for those who will be of our company, but for as many as may be of all the young, and the newly young who are willing to study. It seems to me that there are probably two reasons why we hold this view, not in the first instance commensurable reasons. On the one hand, I think we increasingly feel the need for companionship and for help. I am not here speaking of the patronage of science, which has not been ungenerous or niggardly in the past years, though it may come to be so. I have in mind rather what we all know, that more rapidly than ever before, the sciences have been embodied in new technologies, and that these bring on the scene new powers and possibilities, now a new need, now a new opportunity. These needs and opportunities often are relevant to what in us, and in most men, are the

most deeply held convictions of what is right and good, convictions rooted in a long tradition, and integrally a part of our sensibility. We do not talk about it much, but most of us, I think, are committed to preserving life and health where that is possible. Increasingly, and largely because of the effects during the last centuries of technology and industrialization themselves on its modes, we are committed to limiting, if possible to eliminating, war. We are committed to relieving, to reducing labor and drudgery, and not only the hard labor of the field and the mine and the galley, but the dull labor of the Midlands factory. We are now clearly engaged in a great enterprise testing whether we can live in a world in which war does not play its traditional part, an enterprise in which not only long inherited human institutions, but even older, even other more permanent human attitudes, of anger, hatred, solidarity, self-importance, righteousness, which war has fed, can permit the change. We are in this too deeply, I think, to let the good news or the bad news of the day or month or year affect or limit our hope and, where it is possible for us, our engagement in this great, open, unsettled action of man's history.

With the preservation of life too, and along with it the alteration and automation of work, we are concerned not only with the inadequacy of our institutions, which were framed for a very different world, but with our attitude toward the meaning and value and nature and quality of human life, so largely in our past built on productive work as its foundation. Here in this country we see the mixed fruits of medical and engineering technology first with the young and the old. It is reasonable to expect that they will spread, and that they will characterize many other technologically developed societies. I know of the concern, so well expressed by some of you, that even the saving of children's lives may have created problems with which no one can cope, that have some bearing on the growth and size of the human society.

Though I do not suppose that a thorough knowledge of science, which is essentially unavailable to all of us, would really be helpful to our friends in other ways of life in acting with insight and courage in the contemporary world, it would perhaps be good if in talking with them we could count on a greater recognition of the quality of our certitudes, where we are dealing with scientific knowledge that really exists, and the corresponding quality of hesitancy and doubt when we are assessing the probable course of events, the way in which men will choose and act, to ignore or to apply, or make hypertrophic or nugatory the technological possibilities recently opened. I think that some honest and remembered experience of the exploration of nature, of discovery, and of the way in which we talk to one another about these things, might indeed be helpful; but that is because it would remove barriers and encourage an effective and trusting converse between us, and make more fruitful the indispensable role of friendship. These things are perhaps always easier in a small society. They were perhaps easier a century ago, for us and for many of the countries of Europe. We have a modest part to play in history, and the barriers between us and the men of affairs, the statesmen, the artists, the lawyers, with whom we should be talking, could perhaps be markedly reduced if more of them knew a little of what we were up to, knew it with pleasure and some confidence; and if we were prepared to recognize both the important analogies between what moves us to act and to know, and the extraordinary and special quality of our experience and our communication about it with one another. I

have often thought that with the historic game so grand and so uncertain, we should not dismiss any help, even of that small part which we could play.

The other set of reasons for hoping the young people who will not be professional scientists, and older people who are young in heart, could have a greater scientific literacy and some limited experience, as ours also is limited, is that we know, all of us, that the experience of scientific discovery is a good and beautiful experience, and an unforgettable one. We know that this is true even of little discoveries, and we understand that with the great ones it is shattering. It was on his seventy-first birthday that Einstein said to me, "When it has once been given a man to do some sensible things, afterwards his life is a little different." It seems not really as an act of arrogance but simply human, and not in the purely pejorative sense of the word, to wish these pleasures for as many of our fellows as can have them.

In our world, many things that men do rather naturally, that they have learned to do long, long ago, have become professions, have become part of the market. I think of song and sport and the arts, the practical arts and the fine arts. None of these is without discipline; and although they are very different from those that lead to the sciences, I would be slow to rate them easier. Yet people sing and make sport and practice the arts quite apart from the market, quite apart from a career. It would be a poorer, thinner life without that. Though surely we will not all burst into song, or take to skis, or pick up a chisel or a brush, some of us have done some of these things, and some of us will; and it seems a proper hope that in our education, both for the young, and for those, in growing number, who like us have kept a lifelong taste for it, we do what we can to open the life of science at least as wide as that of song and the arts. Not everybody will want our pleasures, as among us not everyone can taste the other's, and as even we cannot expect an astronomer and a biologist fully to share what each has. We think of this as a high and lovely part of life which, with all its discipline, is still directly responsive to a deep human need. We all know this, and all share it; but each of us, I think, must be free to use his own words to sing its praise, even to describe it.

We may be seeing a time in which war will come to play a smaller and an increasingly trivial part in man's life. I hope that we are. We may be coming to a time in which for growing parts of the world the production of goods will require a much more minor commitment of human effort and life, and the market leave men with a far greater measure of freedom. I hope that we are. For this it will clearly not be enough that we preserve the integrity of our communication and comprehension, either among us, or with our fellows; but this is at the heart of our enterprise, and it is the least we can do.

THE ROLE OF SCIENCE IN UNIVERSITIES, GOVERNMENT, AND INDUSTRY: SCIENCE AND PUBLIC POLICY

By Jerome B. Wiesner

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The announced title of my talk this afternoon was provided for me by Dr. Bronk, who was intent upon making my task just a little bit easier. While I have cast in the mold that he provided, a more abbreviated title would be "Science in the Affluent Society."

We are here this week to celebrate the centennial of our Academy, to honor its founders and the many men who have added to its luster since then. In the previous meetings of this celebration we have listened with excitement to reviews of the many fields of knowledge that compose the body of science. This afternoon it is our purpose to consider the many other faces of science, to review and assess our estate, and to look ahead to the opportunities and needs in science and in the society of which we are a part.

In recent years scientific and technological achievements have been a matter of great public interest as have the many unanticipated problems which the widespread exploitation of scientific knowledge have produced.

It should be gratifying to find, in this centennial year, that the general interest in science is greater than ever before. In the newspapers and other publications, in the Congress too, one finds a growing interest in research and development. There are at the moment several Congressional committees examining the purposes and methods of government-financed scientific and technological activities. While such interest is not new, the point of view seems new. In the recent past, discussions of science often consisted of uncritical praise. Now the situation is changed. Serious questions are being asked and many of them reflect deep-seated concern about the character and purposes of the nation's scientific and technological undertakings. Many also reflect a desire to become more familiar with these processes.

Why has the mood changed so? Has science changed, or was too much expected from science in the past, or have the nation's needs changed? I don't believe that the answer really lies here. What has changed, I believe, is the breadth of motivation for supporting research through the Federal Government. There has been a broadening of emphasis from a primary need to support military development to a wider purpose encompassing the entire spectrum of social needs. The military objective is of great significance, to be sure, but now this is only one of numerous reasons for many of the new research and development activities.

To be specific, while the level of spending for military research and development has remained almost constant during the past 3 years, total federal expenditures for all research and development have continued to rise at an exponential rate. This exponential has a doubling time of about 4–5 years, and the last factor of two involved an increment of approximately $7 billion. Military research and development is still the largest single component of the federal research and development budget amounting to approximately $8 billion of the $14 billion budget for fiscal year 1963.

When the increases in the research and development budget were primarily for

improved military security, they were easier to understand than they are today. In retrospect, our nation was very fortunate—I hope this is not misunderstood—that this incentive for research support did exist following World War II. I doubt that there existed at that time a sufficient appreciation of the general importance of scientific research to have made possible the creation of our present large and very competent scientific establishment on the basis of needs for the general welfare.

Recently in reviewing the political scene, the President observed that there are cycles in the affairs of men. And in science, too, we can observe this fact. We have experienced a heady period of growth, and the time has arrived to review objectives, assess accomplishments, and adjust stressed institutional arrangements to present needs.

We have been through these periods of growth and integration, analysis and reorientation before—many times—during our history as a nation. Starting with the Constitutional Convention, the American people have had a concern for science. And it has always presented a problem to their government. At every period in our history, science has contributed to the development of the nation, and at every step needs of the Federal Government and federal funds have been a major factor in achievements of science. At some point in every historical period the question of the role of the Federal Government in science and the search for the proper form of science organization within the Federal establishment have been burning issues. Though the research efforts involved may appear minuscule by our standards, they were as important to their times as our much bigger programs are to ours. Physical surveys, exploration, standards of time and measure, geological surveys, patent incentives to spur invention, navigation, agricultural science, and public health were among the early issues that involved the government in scientific endeavors.

In his book *Science in the Federal Government*, A. Hunter Dupree traces the evolution of scientific organizations in the government, and here one can clearly see the ebb and flow of enthusiasm for research and technology with passing needs and opportunities.

Most interesting to the contemporary scene is the violent debate which took place over a Department of Science in the 1880's, an argument that was resolved by the Allison Commission, a joint Congressional Commission, when it concluded that the government's scientific establishment and the scientific community in the universities had already grown too complex for such a change in organizational structure.

It is interesting to note that the recommendation for a Department of Science came from a committee of the National Academy of Sciences. It thus appears that committee work for scientists is not a new invention, nor for that matter are government consultants who figure prominently in the early history of Federal science.

One important characteristic stands out above all others in the development of science in the Federal Government, and that is continued growth. In periods of national emergency and in periods of enthusiasm for science, the rate of growth has been faster—during periods of reassessment or preoccupation with other problems such as the great depressions, it has been slower but the averaged slope continues upward. I have examined manpower data which goes back about 40 years, and the steady rate of growth can be seen in them. During this interval, the number of

scientists and engineers has doubled every 12 years. If one assumes that the same rate of growth has persisted since 1800, there would have had to be about 100 individuals engaged in scientific and technical activities, a not too unreasonable number.

I don't cite this history to absolve us of the need to deal with our current problems, but rather to give us some perspective and to indicate that any solutions which we devise today will undoubtedly not be adequate a few years hence. In fact, one need only review the history of the innovations introduced during the past 5 years to see how rapidly the problems change.

The post of Special Assistant to the President for Science and Technology, which I now occupy, was created in 1957. At the same time the President's Science Advisory Committee was made a part of the White House proper. Prior to that time it had been in the Office of Defense Mobilization.

The Soviet Union's launching of the first Sputnik provided the immediate impulse for these moves, but they were also a belated recognition of the greatly increased importance of technical matters in issues of national policy. Dr. Killian, as the first Presidential adviser on scientific matters, rapidly became involved in matters of the greatest national importance involving education, defense, disarmament, space, and international cooperation. In fact, I don't think it is stretching a point to say that the impartial assistance provided by Dr. Killian and the Science Advisory Committee made it possible for the President to arrive at many policy decisions which would have been impossible otherwise.

The creation of the post of Special Assistant provided a means for matching the activities of the Committee to the needs of the President. The full-time Special Assistant was useful to the President in many other ways as well. As a regular attendee at National Security Council meetings, he was in a position to be alert for scientific aspects of a broad range of questions and to provide the President with a technically knowledgeable assistant, with a loyalty to the President alone, rather than to one of the operating agencies, to complement the scientific advice that he received directly from the agencies. During the first year or two, military and space-related problems were of such urgency that they commanded nearly all of the attention of the Special Assistant, his staff, and the Committee. In retrospect this was quite appropriate, since the new arrangements were a response to a failure to fully appreciate or adequately manage a revolutionary technical-military situation. It is clear now—looking back—that the period 1950 to 1960 was truly revolutionary measured in terms of speed, power, and ability to communicate: the three parameters that dominate military capability. The atomic bomb had multiplied deliverable explosive power by a factor of a thousand, and the thermonuclear weapon multiplied this by another thousand. The ballistic missile reduced the reaction time of intercontinental combat from tens of hours to tens of minutes. And the electronic computer made it possible to replace man, whose reaction times are measured in tenths of a second, with automatic devices thousands of times faster. Sputnik resulted in other important organizational modifications; in particular, the Office of the Director of Defense Research and Engineering was established in the DOD, and a civilian space agency was created to provide a focus for the peaceful exploitation of space. The changes in the Defense Department management structure established a scientist or technologist in the top management of the DOD

and contributed much to the rationalization of the technical activities which has occurred in that department.

It is interesting to note in passing that President Eisenhower early turned to PSAC for assistance in unraveling the conflicting information he was receiving regarding the technical aspects of the nuclear test ban which he was attempting to negotiate with the Soviet Union. Starting in April of 1958, the members of PSAC became the President's major source of technical assistance in his attempts to find a mutually satisfactory safeguarded agreement. It was not until the Arms Control and Disarmament Agency was created by this Administration that a continuing full-time effort existed in the Federal establishment to understand the many complicated interacting technical, political, and military issues that must be taken into account when considering an arms limitation or disarmament step. The Science Advisory Committee also assisted President Eisenhower with technical issues involved in more comprehensive disarmament efforts, and several members of the group, including Dr. Kistiakowsky and myself, actually participated in negotiations.

For the first year and a half or so, the Special Assistant and the Committee devoted most of their efforts to security issues, but by 1959 the growth of the research and development programs in the agencies was sufficient to require some coordination and integration, and another step was taken in the formation of the Federal Council for Science and Technology. It was composed of policy officials of the principal agencies engaged in research and development. The Council was to function as a subcabinet for science, to provide advice to the President concerning governmental scientific activities with particular emphasis on problems affecting more than one agency, and to make specific recommendations for the planning, administration, and coordination of federal programs. The Special Assistant also acted as chairman of this group.

When I came to Washington in 1961, I encountered considerable pressure for the creation of a stronger coordination and science policy mechanism at the Presidential level. In fact, there were, and I might add still are, strong proponents of a department of science, complete with Cabinet-level secretary, both in the Congress and among members of the scientific community. The Science Advisory Committee studied the problem for approximately a year and concluded that a department of science, bringing together many research activities now found in the individual agencies of the Government, was not advantageous, but that a need did exist for a more effective and more comprehensive oversight of the federal scientific and technological activities.

Last year an additional administrative mechanism was added to meet this need. The Office of Science and Technology was established by a Reorganization Plan that in effect institutionalizes and makes more permanent those staff resources that had been previously established to advise and assist the President.

Possibly the most important consequence of providing a statutory basis for the scientific activities in the Executive Office of the President is the fact that it enables the Director to appear before the Congress to explain, when possible, the government-wide views of issues, activities, and problems. During the past year I have testified before a great number of Congressional committees and have found this an

enjoyable and useful experience. I hope that the Committee members share my reaction.

Not many years ago in his provocative book entitled *The Affluent Society*, Kenneth Galbraith illuminated the new problems in transition when he attributed many of the economic and social ills of our nation to our failure to appreciate the fundamental changes that technology had made in our industrial society. He pointed out that modern machinery and automation had made it possible to produce all of the consumer goods which our people would ever desire to consume, using a fraction of our total labor force; that following the conventional wisdom existing among economists, we still practiced an economy of scarcity in a society where scarcities need not exist. To use his words:

"The final problem of the productive society is what it produces. This manifests itself in an implacable tendency to provide an opulent supply of some things and a niggardly yield of others. This disparity carries to the point where it is a cause of social discomfort and social unhealth. The line which divides our area of wealth from our area of poverty is roughly that which divides privately produced and marketed goods and services from publicly rendered services. Our wealth in the first is not only in startling contrast with the meagerness of the latter, but our wealth in privately produced goods is, to a marked degree, the cause of the crisis in the supply of public services. For we fail to see the importance, indeed the urgent need, of maintaining a balance between the two."

In this eloquent prose, Mr. Galbraith was pointing to our present dilemma: how to establish a proper balance between satisfaction of our individual wants and the necessary or desirable needs of the community as a whole. There is obviously no simple, or even single, answer to the question of how best to use our available resources. Public support of science has until now largely been exempted from this debate because funds for it were a small part of very large sums of money provided to ensure our military security. The only substantial sums provided for another purpose in recent times were those related to health needs. The unfulfilled social and economic needs around us coupled with one other fact—a leveling off of research and development in relation to gross national product—suggest that the nation faces a significant era of re-evaluation. This process of leveling off explains the reason for many of the current questions. It indicates, as well, that confusions which were of no consequence 10 years ago must be gotten straight today. To the extent that federally supported research and development is justified for social purposes other than national security, it will be judged by other standards, less well-defined, and more controversial as well.

Almost everyone would agree that science is one of the most dynamic forces at work in the world today, that it provides the basis for the technology that permits man to dominate his physical environment, to overcome disease and poverty, in part even to understand himself. In fact, it is this intrinsic dynamic character that stimulates much of the inquiry about research and development today, and is creating the need for new management arrangements. I would like to call to your attention some of the matters of concern that I encounter most frequently.

There is concern about waste of funds and imbalances in the federal programs. There is concern about distortion of our federal activities and our universities. There is worry that the unanticipated but inevitable side effects of new technology

are causing more and more difficult problems which then require further and more expensive remedial actions. Our agricultural surpluses, air and water pollution are good examples. The unpredictable consequences of the widespread effects of the use of pesticides is another, technological unemployment yet another. There is concern that federal research and development expenditures are responsible for unbalanced economic development between geographic areas of the country and between different industries. There is fear also that too large a fraction of the ablest youngsters are being attracted into science and engineering.

Underlying it all is the belief that the whole activity is beyond the comprehension of the individuals in the government who are responsible for it, be they in the Executive Branch of the Government or in the Congress.

Some of these worries stem from real problems, involve the need for policy actions, and should be considered and deliberated. Others stem from misunderstandings which we should strive to eliminate.

For example, research activities and development are frequently not distinguished. They are mixed together and called science. Even most engineers and scientists fail to make a clear enough distinction between activities carried out solely for the purpose of adding to existing scientific knowledge and work that is performed because it may be able to satisfy some practical need. And this confusion is at the root of much misunderstanding among nonscientists who are called upon to make decisions or pass judgments about technical matters. It isn't that most people fail to recognize that there is a spectrum of activities with pure research at one end and hardware development at the other. The problem is rather a failure to understand that the methods and motivations of each are different, and that often research, new knowledge, is necessary in order to achieve a practical goal.

Not only are research activities quite different from development work, but so are their costs. We have estimated that the fiscal-year 1963 obligations for basic research by the Federal Government are approximately $1.4 billion out of the approximately $15 billion devoted to research and development and of this $1.4 billion one-half billion are funds for space science and include the cost of boosters and launching operations. I quoted this figure during a recent Congressional hearing and was later told by Congressman Puchinski that he hadn't realized that basic research was so small a portion of the total. "Maybe," he said, "they were focusing on the wrong problem when they focused their inquiry on basic research expenditures rather than on those for development."

In my view development activities, the creation of useful new devices, should only be undertaken if there is a clear-cut requirement for a new product after it has been developed. This is reasonable, for it is ordinarily possible to make satisfactory predictions about the probable cost and performance of a proposed new device, be it an aircraft, a computer, a chemical processing apparatus, or a nuclear power plant. Consequently, it is also possible to make a decision about the desirability of a given development. Furthermore, because development efforts are generally much more costly than research, one should apply rigorous tests of need before starting new efforts.

In the case of exploratory development and applied research, there is reason to be more venturesome. Here the search is to see if practical applications of new knowledge are possible. For example, there is a vast and varied effort today to

explore possible new uses of the laser, the source of coherent light developed recently, and elements of this work should be carried to the point of demonstrating the feasibility of underlying concepts. Here, too, work beyond such a point should be permitted only if the ultimate capability is needed. These criteria are applicable to all but the basic research segment of the research and development effort. In other words, a basis exists by which administrators and legislators could establish the need for more than 90 per cent of the federal expenditures for research and development. I am not saying that they could or should pass on the technical validity of proposals or judge between competing ways of accomplishing a given objective. This will still be the function of experts, but then the experts will be passing on means, not ends. Corporation executives, government budget officers, and department heads and Members of Congress have traditionally made such decisions with confidence, and with access to scientific and engineering advice; there is no reason why they cannot continue successfully.

But the choices in the field of basic research must be left to the scientists. This is why I place so much emphasis on the matter of distinctions. Even here, others will need to make decisions regarding the over-all level of effort, and if that level is less than is required to support all of the worthwhile research that scientists want to do at any given time, it will be necessary for the scientists to make decisions regarding the support for the different disciplines. Not that this would be easy either. I could write an entertaining book about our efforts to make decisions in the field of high-energy accelerators.

I am very frequently asked whether there are no limits to the growth of research activities, and I find it impossible to give a simple answer to the question. There is no foreseeable limit to the amount of productive scientific research that can be done. On the contrary, I believe that Vannevar Bush was making an understatement when he called science an "endless frontier." It is an expanding frontier as well, if that is conceivable, for on the average each working scientist raises more questions than he answers.

Clearly, the lack of interesting and important work will not set a limit to the extent of our research activities. Many more people have the potential to do effective, creative research than are currently engaged in such work or are studying to become scientists. I will say more about this later. It appears then that the amount of research will be set by the willingness of the country to pay for it. Though we have not passed the point where we can fail to reap increased benefits from increased expenditures on basic research, we have reached the point in time where the rate of growth for the total research and development effort will probably be diminished. I should report a very interesting fact encountered as we prepared for my testimony before the Daddario Committee. Though the actual level of federal research and development obligations rose from $11.2 billion in fiscal year 1962 to $14.5 billion in fiscal year 1963, the percentage of the gross national product for the given years represented by these figures remained constant at approximately 2 per cent. In this climate our basic research activities should be scrutinized with increasing care. Marginal activities should not be tolerated. Here considerable assistance is needed from the academic institutions and from individual scientists, too, for it would be extremely difficult and very undesirable for the government to set standards for academic research and even worse for it to try to police them.

Especially confusing to the lay observer, I find, are the interrelationships between institutions and individuals in the scientific community.

The American scientific enterprise today is a complex one, involving many different associations among the universities, industries, the government, and the individual scientists as well. It is a complicated organism that has very little defined structure. It is the product of evolution in a permissive atmosphere. A single entity in one group may have many relationships with subgroups in the other. For example, a department in a university, or even a single professor, may be engaged in research for several agencies of the government and possibly even for industry. Similarly, a single government agency may support many separate activities through independent and often quite different arrangements within a single institution. Finally, the faculty members of the academic institutions may be found consulting independently for both industry and government. All of this is mighty confusing when witnessed from the outside. There is little wonder that there are frequent proposals to rationalize the system. To be sure, improvement in procedures and more uniformity in practices among agencies is badly needed. But from the point of view of scientific accomplishment, there is great danger that much would be lost if highly structured arrangements were substituted for those we now have. The present arrangements have several important attributes. For the research worker there are a number of different agencies where he can seek support, both speeding up the process of finding support and making it considerably less likely that the prejudice of any single individual or group can dominate the research in a field. Second, it hastens communication, particularly between fundamental research groups and groups in industry or government whose work might be aided by the new knowledge. Third, the frequent contacts between groups results in an atmosphere of mutual understanding, respect, and confidence which are often lacking in situations where formal barriers inhibit them.

Many foreign countries do have much more formal arrangements for the support of research in their universities and industry, more rigidity in relationships between the different sectors of the society, and their scientific enterprise is less effective as a result.

I suspect that most of our colleagues visting us from other countries would urge us to preserve the freedom and rapid communication our arrangements provide. If we are to preserve it, two things must be done. We must be sure that the great advantages of this pattern of operation are understood, and, even more important, that special care is taken by all of the parties involved to ensure that abuses of the freedom thus provided do not occur.

From the point of view of the academic institutions, while federal support for research has made research programs stronger, other problems have been created. First of all, research activities are larger than they could be if the universities still depended primarily upon private philanthropy for support of research, as they did before World War II. Bigness, however, presents problems, and many scientists deplore the fact that often modern research requires large expensive equipment with the concomitant need for team work and a high degree of organization to make effective use of such facilities. Laymen echo these laments without recognizing that many scientific problems today cannot be studied without such tools. Money hasn't created the problem in this case. In other research fields, the size of activities

is related to the level of support, and in them it is true that government support has set the level. As we have already indicated, the motivation for doing this was to ensure that adequate basic research activities existed.

The government turned to the universities for increased basic research because they traditionally have been an important source of new knowledge in the sciences. Such support was of course welcomed by the universities, particularly by the scientists, though many administrators were troubled by the long-term implications of this course. With this support, scientific research in the universities prospered and so did the scientific departments involved. Thus, this activity undertaken for the general public welfare proved very valuable to the academic institutions, too. There was a not wholly anticipated but vital by-product of these actions. The increased level of research made opportunities for more graduate students. If this had not occurred, we would be even shorter of highly trained scientists than we are today. In passing, I might note another popular misunderstanding, that faculty members when engaged in research are not participating in teaching. There is little general appreciation of the fact that the only effective way to train research workers is by the apprentice method. The need for scientific and technical graduates has been so great that the government through the Science Foundation and the National Defense Education Act has awarded fellowships and loans to increase their number further. Increased research activities have created demands for buildings to house the work, which demands have caused a serious drain on institutional resources. Provisions of the Administration's education bill were proposed to mitigate this problem. We are hopeful that the Senate will restore funds for this and other purposes eliminated from the National Science Foundation budget by the House and that the House will accept the educational facilities bill passed by the Senate.

One final difficulty for academic institutions stems from a view now current that an institution doing research with federal funds should make a contribution to the effort by sharing the overhead costs incurred by the work. This is one factor behind current efforts, some of them successful, to limit the overhead rate on research grants to a fixed percentage regardless of what the actual costs may be. Such limitations will save little money and may do much harm to the educational programs of our universities. This is not the only reason expressed for limiting overhead on academic research grants. I have encountered individuals who believed that overhead represented a profit, and, to be sure, a profit of 20 per cent on a government grant would be generous. Here again we see the seriousness of the lack of understanding of fact or purpose.

As the scope of federally financed research activities increases, the problem of proper choice and supervision of grants becomes increasingly difficult. In some academic institutions government agencies must support and monitor hundreds of separate grants, most of them made to individual faculty members. This is a difficult and inefficient task, and the time has come to find a means by which the institutions can accept more of the responsibility for the allocation and use of federal research funds in their institutions. I suspect that neither the funding agencies, the universities, nor the individual researchers will welcome this suggestion, but in my opinion some such arrangement will be necessary if research activities continue to grow. This may prove to be the only possible alternative to the in-

creasingly tight controls that are being imposed on investigators, and the almost impossible supervisory burdens that now fall on some agencies.

To me the most perplexing problem of all is that of reconciling our present basis for awarding research grants based solely on scientific quality and the need and desire to build up the academic and scientific excellence in many parts of the country where it has not previously existed. There is a widely held view that the present system discriminates against areas with modest scientific establishments; that federal funds are used to attract scientists and students from such areas to the large centers, and so, in effect, the rich get richer and the poor get poorer. With the growing realization that economic well-being will be ever more closely linked to technologically based industry, there is a growing determination in most areas of the country not already so endowed to create a strong scientific base—hence the growing resentment and criticism of the manner in which the federal funds for research are allocated.

I do believe that it is in the nation's interest to provide assistance to academic institutions that have the clear potential for becoming centers of excellence in graduate education and research, particularly in geographical areas aspiring to acquire a technological base. Nonetheless, I believe that it would be disastrous to the scientific enterprise if standards other than quality were made the primary basis for the allocation of money for support of basic research.

For that reason, the Administration has asked the Congress to provide some special funds, in the Science Foundation budget and in the funds for the Area Redevelopment Authority, for building centers of research excellence. In this way discretion could be used in providing a base of funds to permit an institution to strengthen its facilities and competence with the objective of becoming competitive for the normal research funds. In this way it would be possible to help the deserving schools and needy areas without destroying the present effective mechanism.

Unfortunately, the House Appropriations Committee did not support this NSF budget proposal.

These issues and many others will undoubtedly be examined by the House committees investigating research and development. The future for all of science will be significantly affected by the outcome.

In this regard, many individuals, many organizations, will inevitably be parties to an inquiry that will be undertaken by the entire nation as well as for the entire nation. It is in this set of circumstances that the National Academy of Sciences could play a highly significant role. I expect that the National Academy Committee on Science and Public Policy will consider many of these issues, too, and their reports will be very important in the continuing discussions.

The next century of service to the nation by the Academy could begin on no more important note than its contribution to a broader comprehension by all sectors of our society—the scientific community included—of emerging implications and opportunities of science in our affluent society.

SYNTHESIS AND APPLICATIONS OF SCIENTIFIC KNOWLEDGE FOR HUMAN USE

By J. B. Fisk

BELL TELEPHONE LABORATORIES

Surveys of the "scientific endeavor" naturally touch on what has happened concerning the *applications* of science in the last century, especially here in the New World. It might be expected that, with our background of social and economic change and the impact of the industrial revolution which had come with full force from Europe about a century ago, we would find in the application of science in America in the past 100 years new patterns and experiences. These could be of special import for future thought and action now that we are in full passage through an Age of Science.

Indeed, there are things to be learned from this perspective of the past century, but much of what we shall now discuss began even earlier, especially in the modes of application of science that were foreseen in western Europe more than three centuries ago. Thus, it is especially timely in assessing this feature of the endeavor of science to see how far we have come and in what directions we have moved with respect to the great original visions of what the practical values of science and technology might be.

The keenest perspective on this is found in the writings of Francis Bacon. Bacon's doctrines were in turn interpreted for a later period by Lord Macaulay. They have been related to our present position by Lord Adrian, the present Master of Trinity College, Cambridge, in his 1961 lecture commemorating the 400th anniversary of Bacon's birth, entitled "Francis Bacon, the Advocate of Science." Lord Adrian reflected on Bacon's classic formulation of the scientific method, and then went on to say, ". . .but Bacon's writing on science had another and greater appeal to his own time, though it concerns an aspect which is now taken for granted. Macaulay and many who have followed him make this by far the most important feature of Bacon's system. He insisted on the great *practical* value of scientific knowledge. The insight which the scientist obtains into nature can and should be employed in commanding nature for the service of man. Macaulay says that Bacon used means different from those of other philosophers because he wished to arrive at an end altogether different from theirs. The end was 'fruit' rather than 'light,' utility and progress in improving the condition of the human race, the good of mankind in the sense in which the mass of mankind has always understood the word 'good.' 'To make men perfect was no part of Bacon's plan. His humble aim was to make imperfect man comfortable.' "

So now we see already a remarkable coordination in the mind and sayings of the philosopher Bacon, so generally accepted as the originator of the modern scientific method, with the idea of the application of science. Indeed, his method, embodying not only theoretical postulates (as from the Greeks), but also experimental trial and verification, is the very essence of modern pure science, and he boldly linked it with a motive of utility. Moreover, Bacon in his *Novum Organum* forwarded principles in this regard which would even outdo the modern project engineer (although our vantage point after nearly 300 years of scientific research and dis-

covery would compel some specification of exact meaning). His statement was ". . .another powerful and great cause of the little advancement of the sciences which is this. It is impossible to advance properly in the course when the goal is not properly fixed. But the real and legitimate goal of the sciences is the endowment of human life with new inventions and riches." Of course, Bacon also valued highly what we have been calling the "light" in science as well as its "fruit," and Lord Adrian puts it all together well in his remark, "It is not cant to put truth above comfort, but most of us would like both if we could get them and Bacon's plan aimed at both, for he held them to be inseparable."

So we have joined historically the essence of the scientific method, whose triumphs we are celebrating once more in this Centennial, with the doctrine of the usefulness of science in human affairs. And our experience of the past century does support the view that these great elements of science—its method and the part of its meaning devoted to human use—are closely interconnected. So I shall try to bring out some examples from various domains of science and technology wherein the synthesis of scientific knowledge for human use seems to have reacted profoundly on *the quality of discovery* itself and especially on *the direction of inquiry* into various fruitful fields of research and invention.

Thus, I should say at the very outset that, under suitable conditions, far from interfering with "science for its own sake," the applications of science seem steadily to be leading us into realms of greater and greater intellectual and even spiritual challenge. Similarly, as the complexity of science rises inexorably on its exponential scale (in terms of total information if not also in terms of basic principles), the vectors of applied science and technology do show directions in which pure scholars may couple to any degree they choose with the human issues and problems of their time. This, too, is not a bad thing for the motivation of men, or for smoothing the path between the ivory tower and public plaza.

My part today is especially to discuss the *ways* in which this synthesis of scientific knowledge for use seems best to be achieved. At one stage, in our Academy's youth, it was said that professional engineering, coupled with a diligent search for invention, sufficed as ways to attain this objective. The successes of those methods are well known, especially in such cases as the vital development of the Bessemer process for steel making (including the concurrent patents of William Kelly of the Kelly-Pneumatic Process Company in this country!). The era of Edison was marked by this mode of applying new knowledge. Edison's conversations, as quoted by George Parsons Lathrop, develop this concept of the relations of discovery to invention. He said, "Discovery is not invention and I dislike to see the two words confounded. A discovery is more or less in the nature of an accident. . . . Goodyear discovered the way to make hard rubber. He was at work experimenting with India rubber, and quite by chance he hit upon a process which hardened it—the last result in the world that he wished or expected to attain. Bell's telephone was a discovery, too. He was engaged with the possibilities of sending sound waves over a telegraph wire, and found an invention by which this could be done. Then, by accident, it was discovered that articulate speech could be sent out over the wire—and there was the telephone. But Bell did not set out to make an instrument by which talk could be transmitted, and therefore I say he discovered instead of invented the telephone. In a discovery there must be an element of the accidental,

and an important one too; while an invention is purely deductive. . . . In my own case but few, and those the least important, of my inventions owed anything to accident. Most of them have been hammered out after long and patient labor, and are the result of countless experiments, all directed toward attaining some well-defined object." Edison said then, concerning the very difficult work on the electric light, "And yet, through all those years of experimenting and research I never once made a discovery. All my work was deductive, and the results I achieved were those of invention pure and simple."

Thus, we see that the specific goal-setting of engineering, which is of course vital to the magnificent successes of that profession, has long been differentiated from the method of discovery. Yet in the growing application of science for human use there have arisen increasingly satisfactory combinations of the subtle elements of scientific discovery, and of the hard-driving, problem-solving techniques of engineering. This is foreshadowed by Langmuir's remarks, in his essay on "Fundamental research and its human values," concerning his discovery of the gas-filled electric light bulb. He said, "I want to show you how. . .the practical result could hardly have been reached in a laboratory in which the workers were assigned definite work. . . ." But Langmuir's research, carried out in a "practical" environment, led to a succession of triumphs.

What has been the key factor in merging these diverse attitudes, so that Bacon's original aspiration of science for the betterment of the human condition is being increasingly realized? I submit that, curiously enough, this element is frequently ignored in the highly personal evolution of the body of science itself. It is an institution of men, a structural community of experts. In the face of the universally accepted precept that scientific (and almost all other scholarly) achievement is the result of individual effort exerted by particular, gifted minds, it is striking that a community of effort constructed as an institution of men is the basis for the most spectacular progress we have made in the application of scientific knowledge for human use. You may at once indignantly reject this finding, and ask: How can you say that when the history of applied science is also that of individual genius—as in Pasteur's vaccines, in Bell's telephone, in Edison's light bulb, in Hall's aluminum process, and indeed in Chadwick's neutron? Truly these were the results of individual, personal insight and ingenuity. So I would quickly say that I am not talking about teams, as diligent and efficient as they have been in the application of knowledge in many vital areas such as synthesis of antimalarials and antibiotics, the evolution of microwave technology and radar, the engineering of nuclear energy, and so on. Rather I am saying that the best application of science and the synthesis of new knowledge turns out to be by a community of gifted people working intimately but independently, with each free to follow his own mind. Such a group constitutes the essential mechanism to bring together the diverse elements, many disciplines and kinds of learning from the most abstract mathematics to the most concrete mechanics and design.

Indeed it is interesting that, in the historic advance of science particularly in the past century, quite separate disciplines such as physics, chemistry, biology, and so forth, were formed. Certain very distinct compartments were created even within those disciplines, such as organic chemistry, nuclear physics, molecular biology, etc. These divisions arose and represented domains in which, by bold simplifica-

tion and postulate, scholars had been able to understand certain parts of nature. This naturally was also the way to teach this understanding, and so these classifications were retained and have become classic parts of the school and university structure.

But, of course, the needs of man at times are relatively unconnected with these particular categories of human knowledge. They are divisions of knowledge, in which the most fruitful experiments and the most revealing theories could be exercised. For a long time this kind of classification extended also into engineering itself, as revealed by the designations: mechanical engineering, electrical engineering, and so on. More recently, terms like "industrial design" have become prevalent, and these begin to recognize the "human use" features of the profession. Medicine or medical science was probably the earliest recognition of such technical processions devoted to human needs, but itself was separated (and some say it still is) from the classical categories of biology, chemistry, and physics, as well as statistics and other mathematics, which provide its new and growing resources.

So this notion of combination of the sciences, or what is resonantly termed interdisciplinarianism nowadays, representing the free interaction of experts in a very wide range of scientific endeavor, is an institutional quality which has arisen almost entirely in the past few decades or at least half a century. It has also arisen very largely because of a new coupling of science with the human needs. Not surprisingly, such a structure is at last being found to be of great merit in approaching refractory problems of understanding nature for its own sake. There is a trend to have groups of many formally separate parts of science clustered around great nuclear accelerators or in genetics laboratories. And, of course, in an increasing variety of national scientific endeavors, such as space exploration, nuclear energy production, oceanography, and assaults against cancer, coronary disease, and other human afflictions, this combination of diverse specialities and its institutional cultivation are appearing.

Hence, it is useful on this occasion to recognize the relative newness of our experience with this kind of scientific and technical endeavor and to try to trace some of its origins and to suggest some of its projections. Strikingly, the philosopher Alfred North Whitehead, in his book *Science and the Modern World*, saw some of the essence of the matter in comments he made 40 years ago about the 19th century itself. He said, "What is peculiar and new to the [19th] century, differentiating it from all its predecessors, is its technology. It was not merely the introduction of some great isolated inventions. It is impossible not to feel that something more than that was involved. . . . The process of change had been slow, unconscious, and unexpected. In the 19th century, the process became quick, conscious, and expected. . . . The whole change has arisen from the new scientific information. Science, conceived not so much in its principles as in its results, is an obvious storehouse of ideas for utilization. . . . Also, it is a great mistake to think that the bare scientific idea is the required invention, so it has only to be picked up and used. An intense period of imaginative design lies between. One element in the new method is just a discovery of how to set about bridging the gap between the scientific ideas and the ultimate product. It is a process of disciplined attack upon one difficulty after another. . . . This discipline of knowledge applies beyond technology to pure science, and beyond science to general scholar-

ship. It represents the change from amateurs to professionals. . . . But the full self-conscious realization of the power of professionalism in knowledge in all its departments, and of the way to produce the professionals and the importance of knowledge to the advance of technology, and of the methods by which abstract knowledge can be connected with technology, and of the boundless possibilities of technological advance—the realization of all these things was first completely attained in the 19th century. . . ."

Now this attainment, referred to by Whitehead, sounds as though the scheme we are emphasizing as the principal way by which new knowledge is synthesized in science for human use was indeed old rather than relatively new. Actually, Whitehead had observed, specifically in Germany, certain highly developed forms of organizing to achieve particular technical ends, especially in the chemical and metallurgical industries. He shrewdly discerned the general outlines of the methods. I think he did not mean to say that there was much use of combinations of experts from various fields but rather that groups of experts of some sort were the basis of this revolutionary professional organization and use of new knowledge from science.

In our next considerations of how these communities of experts may be cultivated best to extend the synthesis of knowledge for human use, we should recall that James Clerk Maxwell, in delivering the Rede lecture nearly 80 years ago, chose to talk on "The Telephone." Apparently what interested him was Bell's ability to bring together diverse sources of scientific and technical information, and even more to appreciate the points of view of various specialists in the relevant fields of electricity, acoustics, physiology, and others. Already, then, Maxwell saw the opportunities for combination of many new kinds of knowledge into useful results. Thus, he said: "I shall, therefore, consider the telephone as a material symbol of the widely separated departments of human knowledge, the cultivation of which has led, by as many converging paths, to the invention of this instrument by Professor Graham Bell."

"For whatever may be said about the importance of aiming at depth rather than width in our studies, and however strong the demand of the present age may be for specialists, there will always be work, not only for those who build up particular sciences and write monographs on them, but for those who open up such communications between the different groups of builders as will facilitate a healthy interaction between them." This was said 80 years ago.

Now we have found in the succeeding years in attempting, for example, to carry on the enterprise initiated by Bell's discovery of the telephone, that the cohesive element which provided this healthy interaction of Maxwell is a common and a tenable set of objectives. Curiously enough, it is not a case where, in the history of electrical communications, for instance, theories or important discoveries have themselves formulated the objectives of the enterprise. Rather, clear, simple, technical thinking about the human needs suggested goals of such value and challenge to the mind that communities of scientists and engineers were interested and willing to combine all sorts of skills and learnings in order to advance toward these goals. Thus the idea of nationwide and continent-wide telephony, in which any person anywhere in the nation could, through his own telephone, speak to anyone anywhere else, was, at the time it was put forward by Theodore Vail, a highly visionary statement. Yet it was a simple principle, meaningful technically, which has consistently

and progressively energized the range of effort in our telephone laboratories, beginning with the exploitation of the qualities of electromagnetic induction found by Joseph Henry as embodied in the loading coil, and extending through to the creation of the vacuum tube by H. D. Arnold, the discovery of negative feedback by Harold Black, of the coaxial cable and carrier principles by Affel and Espenschied, advances in information theory by Claude Shannon, the invention of the transistor by Bardeen, Brattain, and Shockley, the discovery and exploitation of the traveling wave tube by Kompfner and Pierce, etc.

Along this route toward Vail's goal which, of course, has been somewhat elaborated into the notion of worldwide telephony and electrical communicating now, have arisen many byways. Many of them were tempting byways, such as the implementation of the first radio broadcasting, the realization of sound for moving pictures, the demonstration of full color video, even the discovery by one engineer working on telephone transmitters of the principles now embodied in the universally employed diaphragm fuel pump for automobile engines. An issue of importance for this gathering is that it was decided, each time with considerable agony, *not* to pursue these byways to the exclusion of the main pathway toward the central goal. There seems to be a good deal of power in this policy with respect to our central theme of cultivating communities of experts in science and technology so that they show optimum effectiveness in meeting the needs of man. There is the especially human tendency for experts to want to see developed their own special discoveries. Naturally, the chemists who found in the telephone laboratories a way to make laminating plastics out of previously unusable but highly stable and efficient polymers would like to see a generalized application in the form of materials and laminate manufacture with usual product development, etc. The physicists discovering electron diffraction in the telephone laboratories might even be glad to see a venture into instrument making for the widespread use of this technique. The engineer and acoustics expert finding ways to make sound tracks on moving pictures in the telephone laboratories would like to see a major venture into the mass entertainment–moving picture field result from this discovery. Innumerable examples could be cited and could be multiplied in many other laboratories.

Now the point is that as soon as these diverse paths of technical exploitation are opened up, and indeed they are often most attractive to managements in terms of quick profits and rapid invasion of a new market (which offhand seems importantly to reflect a new human need), nevertheless as soon as they are opened up, each of the community of experts begins to think, "Now it is my turn to make something, some sort of discovery or invention or new level of understanding which will send our institution off into my particular direction." The great goals become fragmented, and the original purpose of being able to turn a marvelous diversity of knowledge from all the independent and separate sciences and arts into a common, generally oriented effort toward major overriding goals for human uses, is badly obscured.

So I assert that the chief tenet for assembling scientific knowledge for human use is the formulation of objectives which are (*a*) sufficiently important, (*b*) suitably broad, and (*c*) technically meaningful so that they will provide a vast template of opportunities for the specialties and for the individualities of creative scientists and engineers, to inscribe the over-all effort without providing diversions. (The

danger of these diversions, incidentally, may also apply strongly to our present national preoccupation with side benefits, or "fallout," both economic and social, from major national exertions.)

Also, the quality of being sufficiently important is one which technical goals for exploitation of scientific knowledge do not easily specify themselves. As other speakers in this session have shown, there are also things which are only with great difficulty specified by the judgments of busy governments and bureaucracies. There is, however, particularly in our system of free enterprise, which has flourished so strongly in the century we are celebrating, a curiously pervasive scale of values. It is competition in the public market place. While the scientist and the engineer will never feel that his public understands all the values or rates properly the importance of his technology, nevertheless there is an increasing discernment by the public of the meaning of science and engineering for the betterment of man. There is a very strong indication that the kinds of human needs for whose satisfaction humanity is willing to pay give useful guides to major goals.

Thus, I have purposely touched on questions of economics, of politics, of statecraft, of philosophy which, of course, have been and will be treated by others.

But we come back always to a few basic precepts, which can lead us to ever-mounting advance in the synthesis and applications of scientific knowledge for human use. Paramount is this doctrine of the right objectives—important, broad, meaningful—which will provide both force and direction for progress in the uses of science. And according to the ideas we have forwarded, the generation of new knowledge, indeed the vigor of science itself, will gain from this process of seeking to apply it. For the great thing we have learned, especially in the past century of modern science, but even as it was forecast by Francis Bacon, is that scientific matters related to human needs can be and very often have been the most challenging and "interesting" questions of all science.

While the great upsurge in the study of genetics and molecular biology, indeed of the vital process itself as reflected in abnormalities, such as malignancy, are current examples, there are many earlier cases: Planck's interest in luminescence leading to the quantum theory; communications and radar efforts leading to microwave spectroscopy; the ceaseless quest for the sources of energy, which apparently underlay much of the basic curiosity about nuclear forces. Over and over we see that high intellectual as well as practical values have been provided where it was possible for gifted minds to follow the kinds of goals we have tried to characterize.

So it can be in the future that science and its companion, engineering, may make both noble and benign the lot of man on earth and in his reaching toward the heavens. But we must take thought to form the aims and to construct the institutions which extend what we have already learned about the synthesis and application of scientific knowledge.

There has been a feeling, especially in the past decade, that science is getting too big for man and that we would either be destroyed by malign effects produced by it or be confused and eventually suffocated by the sheer mass of data and infinity of concepts which it would impose on our culture. The view with which we conclude here is a vastly happier outlook. It reveals, on the one hand, that only the full satisfaction of all human needs (a situation not immediately in prospect)

should distract us from setting goals for human use of such quality as to absorb constructively and beneficially any and all of the scientific findings to come.

On the other hand, we see the culture of science itself gaining from this endeavor, because the way of seeking these goals will demand an ever-increasing combination and even a fusion of scientific skills, from what have been called, up to now, "different" sciences. While the endless variety of approach to science must be zealously preserved, these differences between sciences will grow less and less. Many of us believe that this change itself will aid emergence of great unifying principles. (Think, for instance, of information theory, of the coding of electrical signals, of the coding of nerve impulses, of the coding of bases in nucleic acids and of their influence on protein configuration.) For those in science and engineering who seek to solve a worthy problem, and to reach an end which is important, broad, meaningful, are finding ways more and more to bring all kinds of minds and skills to work together.

SCIENCE AND THE SATISFACTION OF HUMAN ASPIRATIONS

By I. I. Rabi

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Ladies and gentlemen, here we are at the end of a long day after almost a week of much talk. Our next appointment is not till 7:30 this evening. I therefore have plenty of time to devote to my topic; however, my audience would decay exponentially. I hope not to detain you for long, for it is well known that only very few souls can be saved after half past three in the afternoon.

In this conference we have heard from some of the most eminent men of science in the land. Day after day the mysteries of life were laid bare, and antecedent to life the structure of matter and, indeed, of the universe were presented in dramatic and fascinating clarity. Although no attempt was made to make a real interdisciplinary connection between the various disciplines, nevertheless, the juxtaposition of topics did a very great deal to show us the essential unity of the scientific disciplines, however different their techniques. The very first day we went in progressive steps from the origin of the elements to the origins of life. In the second day we went from the almost philosophical consideration of the organization of the laws of nature to the organization of living matter. This afternoon, turning to less immediate questions, we have had a general overview of science from the aspects of communication and application both to industry and to public policy and welfare. We listened to great wisdom.

It is now my turn. The task assigned to me was to address myself to science in its more intimate relation to the individual: science and the satisfaction of human aspirations. The drives which cause men to become interested in science are almost as various as human personality. Science could not happen without a range of personalities and cultures. We see the interplay now of cultures in the successive contributions of Egypt, Babylon, Greece, Alexandria, Rome, Arabian-Italian Renaissance, and in the modern era Japan, America, and China, which should

perhaps be all by itself. There is the collector and classifier who may interest himself in stamps, books, pictures, or, on the other hand, in phases of botany, geology, astronomy, or even in parts of physics such as spectroscopy. We should not use the term "mere" with respect to these activities. High talents of intellect and insight can be devoted to these aspects of sciences. The hunt for a new species of plant or animal, a new spectral sequence, a new collection or system of galaxies can be both exciting and demanding. Ingenuity, persistence, and what may be called luck are necessary for success in these endeavors. The born hunter and alert observer will find success and satisfaction in this phase of science. We would be nowhere without this type of individual.

Basically, this element of science satisfies more immediately the desire to discover and to know the facts of nature. Nature with tremendous variety and charm captures the fancy and the spirit. To one person a fact is just a fact and of no further interest. Such a person may become a mathematician or a logician, but he will never be a true poet or scientist. The scientist, the experimental scientist at least, shares with the poet and artist a feeling for the value for the immediate and the empirical face of nature. The geologist loves his bright and shiny stone, his curious fossils, just as the physicist can never cease to be charmed by a spectrogram or the delightful paradoxes of the motion of a spinning top.

This aspect of the pursuit of science satisfies a basic desire or aspiration just to know, to find out, or perhaps make order out of the otherwise chaotic jumble of immediate experience. It is an aspiration shared with all mankind but more with youth and childhood than with adults. In this sense scientists are just children who never grew up, who never lost the nagging urge to ask how, why, and what. Like children, who in all innocence and high excitement bring a dangerous spider into the house and frighten the wits out of their elders, the scientist emerges with a smallpox vaccine or an atomic bomb.

There is another facet of human aspiration which contributes to the various faces of science. This is man in his aspect of the maker or doer. Again this aspect is strongest in childhood but persists longer into adulthood since its immediate use is obvious. The use of tools, the arts and crafts, are the hallmarks by which we rank prehistoric and primitive civilizations. Of course, arts and crafts are not yet science, but share with science the manipulation of nature. The one is to satisfy the desire for material needs of food, shelter, decoration, and of course armament. This is the usual, the normal—the sort of thing which is immediately understood by men of maturity and judgment such as we find in Congress. The other, the less practical but in the end more powerful, is to manipulate nature not for immediate or material ends but for the purpose of providing new knowledge or the tools which could provide new knowledge. This is the method of science.

It is rare that this aspiration for discovery is sufficiently understood by any community to the degree of actually providing funds for this purpose. Either the curiosity of childhood is soon lost (perhaps this change may have an actual survival value for the race) or somehow those who possess the gift lack the ability to communicate the deep meaning, the excitement, and the satisfactions of scientific discovery.

This question should be of the greatest concern to our scientific community. Unless the public shares in our aspirations and our satisfactions in the scientific

enterprise, the pure scientific impulse will always have to be diluted and even distorted with immediate ends, which in the last two decades have been primarily military.

I see nothing ignoble or degrading in the application of science to the defense of one's country—quite the contrary.

On the other hand, when the support of science is tied both administratively and by public interest directly to military and other purely practical uses, one begins to feel that somehow we as scientists have failed to arouse either interest or understanding in the public mind. We have failed to satisfy the aspirations which they share with the scientist by taking them with us as we go further along the road of scientific accomplishment. This community of interest which we have with the youth of the country in considerable measure does not extend to the adult population. Surely, science is not only for children young and old.

I will not infringe further on what I have hoped would be Dr. Oppenheimer's domain of science and communication, but go on to still another facet, perhaps the most significant of the scientific adventure, or scientific movement, or, as I prefer to call it, the scientific culture. In this, we share with the poet and artist the delight in immediate empirical experience with its aesthetic, emotional, and intellectual values, but we go further, not to express this experience in the language of the heart, but in the prose of the catalogue, finding similarities in differences and presenting the results as an intellectual structure which can inspire pleasure and interest. We share with the practical world the manipulation of nature but not principally directed to useful ends or ends which are said to be useful. Our ends go further, but we do share with the artisan and the engineer the pleasure of invention of novel combinations to achieve what had hitherto been difficult or impossible.

Our goal is a sort of bootstrap operation to utilize the tools of present knowledge to gain new knowledge, knowledge which we could hardly have foreseen or imagined. In these two illustrations I hoped to show that in our interaction with the world outside ourselves the scientific aspirations and satisfactions are basically aspirations which are shared by all. The scientists' satisfactions come in a special form which expresses itself in the desire to broaden and deepen our knowledge and understanding of all phenomena, but whereas the rest of mankind concentrates on man, his feelings and desires, the scientist tries to see the world as it really is or might be shorn of man's perhaps excessive preoccupation with himself. Clearly this is a quest which can never come to an end. Scientific curiosity will never be satisfied because it will never reach its goal to know all and understand all.

Such a goal and such an adventure will hardly satisfy the more prosaic and limited aspects of our human nature, but it nevertheless has a nobility of a kind which in other fields has called forth some of the greatest manifestations of the human spirit.

The third aspect of science which I wish to explore with you, and which may be as I suggested the most significant, is one which we share with the humanities and with religion. Except for some periods of uneasy truce, science and religion have always been in conflict. Since the time of Galileo this conflict has sharpened. Many able men, both from the side of science and of religion, have assured us that there is no conflict between the two. On closer examination, it is apparent that the synthesis

or bridge which they try to establish results only in a devaluation of both aspects of a powerful urge of the human spirit.

The urge to comprehend the visible and invisible universe and to find man's place within it is common to both science and religion. The conflict between science and religion is, therefore, more in the nature of a civil war between two parties with the same ultimate aims of comprehension and of submission to a higher order of knowledge and of insight.

In these matters religion has always taken the lead. Questions about man's place in the universe and his origins had to be given answers in each generation.

The ancient Hebrews could not wait for the discovery of the neutron and the development of the theory of stellar evolution, or for Darwin, Morgan, Crick, and Watson to explain the variety of life and the origins of man and of the universe.

The noble opening lines of Genesis cannot fail to move the most prosaic scientist even today. By means of dramatic imagery and lofty poetic insight, religions have provided world systems more or less complete which gave immediate satisfaction to the yearnings of man for order in the world and guidance in his life. They gave a release from certain fears, although they sometimes substituted others for those displaced. The great human quality of faith was always a basic prop to these religious structures. Religion and religious systems to be fully effective had to become established in law and custom, and in a certain sense their statements had to come to be regarded as self-evident.

Compared to the eagle flight of religious thought, science is more like the humdrum earthbound bulldozer. Where it has passed anyone can follow. Whereas religion is aristocratic and hierarchical, science is democratic and leveling. After the bulldozer has passed, a tangled jungle or many beautiful gardens and buildings enshrined in history and sentiment may be destroyed, but the ground is ready for newer and perhaps even more beautiful interesting cultivation, or perhaps not. In any event, a newer generation gets a new start.

It has often been said that science gives man knowledge but does not tell him what to do with it. These prescriptions and values he is supposed to get from religion or from the so-called humanities. To borrow a phrase from a great scientist, Enrico Fermi, "This is not very true." The great writings of the humanist and holy religious writ can do much to incite men to noble and charitable action as well as to acts of folly and cruelty.

Science can make no such claim. What science seeks to do in its limited way is to delineate the results of action through psychology on the individual himself, through the behavioral sciences on the others, and, above all, to present a choice of means, leaving the decision to an informed act of "free will" insofar as the term still has a meaning. Only those who do not care to make their actions their own will say that science does not contribute to values.

The conflict between science and religion, between science and the humanities, therefore remains. The latter must always claim more than it knows and, therefore, must always retreat and qualify as science advances. "Don't insult me with facts" is the hurt expression of punctured pretensions. The true humanist and religionist welcomes scientific advance because it also allows him to advance his cause with deeper understanding. The Holy Fathers of the Roman Catholic Church have made this point very clear.

In contrasting science with the humanities and religion, the more pedestrian elements of the relentless march of scientific discovery have been emphasized. Actually science does not march in battle order toward a predetermined goal. This would be a contradiction in terms.

Scientists traditionally are free, untrammeled, and individualistic. Each sets his own goals following his interests. Such co-ordination as there is comes out of the nature of the subject matter and out of the tradition of the discipline; attempts to interfere, direct, or guide this freedom, as in some countries with overplanned societies, result in inefficiency and frustration of the creative urge. Scientists are well aware that they are prone to error. The observer in his laboratory knows full well that he can easily misinterpret his observation or miss the essential fact. The bold speculator can become so enamored of the beauty and sweep of his hypothesis that he may take it as an end in itself. It must be true, he feels, because it is so elegant. However, the court of highest appeal, which is nature itself, is relentless, and error cannot long survive.

Therein lies one of the greatest appeals of science, an appeal which makes it capable of satisfying one of the greatest of human aspirations—to be a member of a community which is free but not anarchical. Science possesses an infinite variety of limited goals but in the end marches toward a limitless horizon. It consolidates its gains but does not rest on its laurels. Members of this community possess an inner solidity which comes from a sense of achievement and an inner conviction that the advance of science is important and worthy of their greatest effort. This solidity comes in a context of fierce competition, strongly held conviction, and differing assessments as to the value of one achievement or another. Over and above all this too-human confusion is the assurance that with further study will come order and beauty and a deeper understanding.

One cannot close a discussion like this without bringing out one of the greatest rewards of the pursuit of scientific discovery. It comes accidentally and is often a matter of luck rather than the result of planning. It may come in an illuminating flash of insight or in the course of an experiment such as Rutherford's when he saw his alpha particles scattered through large angles, or Anderson's when he saw an electron track moving the wrong way and realized he had a positive electron, or Yukawa's when he saw that a supposed particle could account for nuclear forces. Although scientists don't write about these moments of exultation and ecstasy so different from the everyday routine of research, these fleeting visions can in one flash reward one for years of patient and exhausting work. At these times the scientist is filled with profound awe and humility that such wonders should be revealed through him. There is a quality about science, or rather about nature, which is always miraculous in its originality. To obtain a glimpse of this wonder can be the reward of a lifetime. This itself can be the sufficient satisfaction of the aspiration which makes scientists scientists.

At this point, at the end of my presentation and the end of this program, I can only wish the Academy and all mankind a century of achievement as great as the century which has passed in the life of our beloved Academy.

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